551,789

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 October 2004 (14.10.2004)

PCT

(10) International Publication Number
WO 2004/087173 A2

(51) International Patent Classification[7]: A61K 31/7032, A61P 29/00, 3/06

(21) International Application Number: PCT/CA2004/000375

(22) International Filing Date: 12 March 2004 (12.03.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/404,095 2 April 2003 (02.04.2003) US

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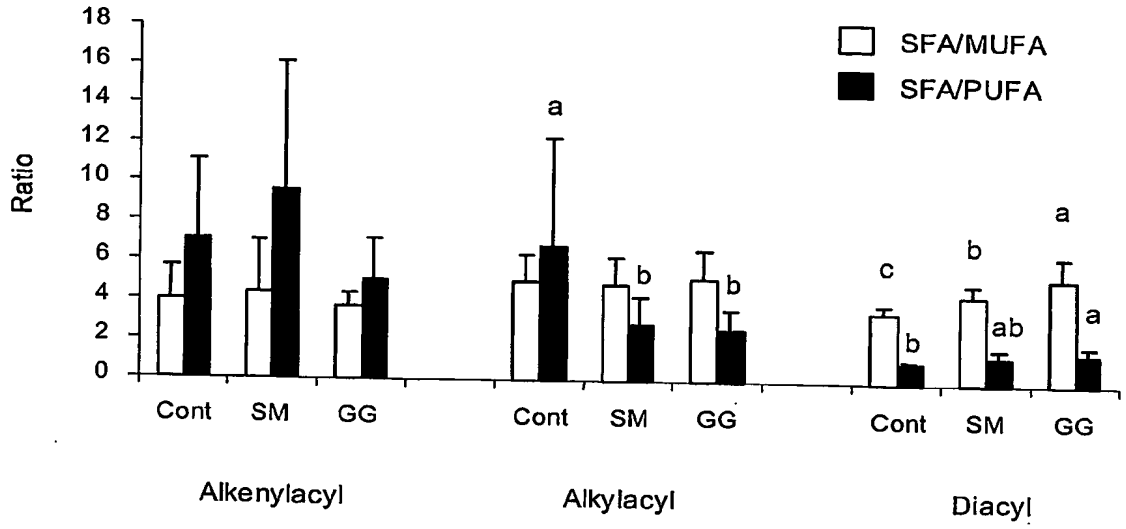
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

[Continued on next page]

(54) Title: FORMULATIONS FOR MEDIATING INFLAMMATION AND FOR REDUCING BLOOD CHOLESTEROL



(57) Abstract: The invention provides formulations for mediating inflammation and for lowering blood cholesterol. For example, inflammation of the intestine, retina or neural tissues, may be mediated. Further the formulations are effective in decreasing blood cholesterol absorption. The formulations comprise at least one ganglioside, which may be selected from the group consisting of: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid. The invention further provides a method of treating or preventing inflammatory diseases by delivery of at least one ganglioside to a subject in need thereof, and a method of reducing blood cholesterol in a subject be delivery of a ganglioside-containing formulation. The formulation of the invention may be used to supplement foods or liquids, and may for example be used in preparation of infant formula or foods.

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FORMULATIONS FOR MEDIATING INFLAMMATION AND FOR REDUCING BLOOD CHOLESTEROL

FIELD OF THE INVENTION

[0001] The present invention relates generally to a formulation for mediating inflammation, such as inflammation of the intestine or other tissues such as retina, retinal tissues or neural tissue.

BACKGROUND OF THE INVENTION

[0002] Gangliosides are specialized sialic acid-containing glycolipids abundant in the outer region of the neuronal lipid bilayer and intestinal brush border. The intestine contains a relatively high amount of ganglioside (as much as 7% of total lipids (Christiansen *et al*, 1981; Forstner *et al.*, 1973). Change occurs in the composition and molecular structure of gangliosides during intestinal development (Bouhours *et al.*, 1983; Glickman *et al*, 1976). Total intestinal lipids are comprised of 25 to 35% sphingolipids, including gangliosides and sphingomyelin (Christiansen *et al*, 1981; Forstner *et al.*, 1973), and microvillus membranes are more enriched in gangliosides than the plasma membranes (Forstner *et al.*, 1973).

[0003] Gangliosides are located at the surface of the cell membrane with the hydrophilic oligosaccharide chain extending into the extracellular space. Glycosphingolipid constitutes approximately 20% of the brush border membrane lipids (Forstner *et al.*, 1973). The dominant ganglioside is GM3 which is 7 times more concentrated in the neonatal compared to adult intestine of rats (Bouhours *et al.*, 1983). The specific physiological roles of gangliosides are poorly understood, however, studies showed that gangliosides provide binding sites for a wide range of pathogens including viruses, bacteria and fungi (Holmgren *et al.*, 1985; Kyogashima *et al.*, 1989; Laegreid and Otnaess, 1987; and Rolsma *et al.*, 1998). For example, ganglioside GM3 acts as a natural receptor in pig small intestine for rotavirus (Rolsma *et al.*, 1998) and the enterotoxigenic bacteria *Escherichia coli* (*E. coli*) K99 (Kyogashima *et al.*, 1989). Ganglioside GM1 in human intestine (Holmgren *et al.*, 1985) and in human milk (Laegreid *et al.*, 1987) also provides receptors

for enterotoxin of *Vibrio cholerae* and the heat-labile *E. coli*, thereby acting as a physiological barrier for protection against these enteric infections.

[0004] Previous studies showed that gangliosides exist in clusters in the plasma membrane forming glycosphingolipid enriched domains and that these domains are the preferential interaction sites between target cells and pathogens (Karlsson, 1995). Preterm newborn infants fed ganglioside supplemented formula at a concentration of 1.43 mg/100 Kcal, were shown to have significantly lower numbers of *E. coli* and *bifidobacteria* in the feces (Rueda *et al.*, 1998).

[0005] During early development, important morphological changes occur in the total and relative amounts of gangliosides in neuronal tissues of the brain and retina (Asou *et al.*,1989; Baumann *et al.*, 1976; Daniotti *et al.*, 1994). One of the primary roles of gangliosides is activation of neuronal cell differentiation and proliferation (Ledeen *et al.*, 1998), influencing synaptogenesis and neuritogenesis (Byrne *et al.*, 1983; Svennerholm *et al.*, 1989) and offering protection against neuronal injury (Guelman *et al.*, 2000; Mohand-Said *et al.*, 1997). Functions in the intestinal mucosa involve toxin receptors of bacterial and viruses (Thompson *et al.*, 1998; Rolsma *et al.*, 1998) and immune activators (Vazquez *et al.*, 2001). Radiolabeling studies have shown that exogenous gangliosides and sphingomyelin are hydrolyzed by enterocyte membrane-bound enzymes such as sphingomyelinase and/or ceramidase (Merrill *et al.*,1997; Schmelz *et al.*, 1994; Nilsson, 1968). Metabolites such as ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate are transported into enterocytes and reutilized in synthesis of gangliosides or sphingomyelin or both (Merrill *et al.*,1997; Schmelz *et al.*, 1994). Since gangliosides and sphingomyelin are incorporated into lipoproteins and chylomicrons (Hara *et al.*, 1987; Merrill *et al.*, 1995), dietary gangliosides, sphingomyelin and/or their intestinal metabolites are likely to be transported throughout the body to affect sphingolipid biosynthesis in other organs (Vesper *et al.*, 1999). Studies have suggested a possible interaction between sphingolipids and phospholipids (Merrill *et al.*,1997; Schmelz *et al.*, 1994; Ogura *et al.*, 1988). Sphingosine-1-phosphate is metabolized into ethanolamine phosphate and hexadecanal, both prerequisite materials for phospholipid synthesis (Merrill *et al.*,1997; Schmelz *et al.*, 1994). Radiolabeled ganglioside

[^{3}H]sphingosine-GM1 when injected intraperitoneally into mice was incorporated into hepatocyte phospholipids in the EPL form (Ogura *et al.*, 1988).

[0006] ***Ether Phospholipids.*** To date, there has been no research investigating whether dietary gangliosides can be used for synthesis of EPL in the intestine or influence EPL synthesis in neuronal tissues. EPL have an ester linkage at the sn-2 position, but have an ether linkage, either to an alkyl or alkenyl group, at the sn-1 position. EPL tend to be enriched in mammalian intestinal and neuronal cells (Paltauf, 1972). One type of EPL known as plasmalogens (a group of 1-O-alkenyl-2-acyl-glycero-phospholipids), accounts for about 75% of ethanolamine phosphoglycerides (EPG) in myelin of rat brain, 65% of EPG in human brain (Horrocks, 1972) and 12% of EPG in rat intestinal mucosa (Paltauf, 1972). High content of EPL may contribute to maintenance of cell integrity and function (Alonso *et al.*, 1997; Bittman *et al.*, 1984; Diomede *et al.*, 1993; Honma *et al.*, 1981; Mavromoustakos *et al.*, 2001; Oishi *et al.*, 1988; Paltauf, 1994; Principe *et al.*, 1994; Seewald *et al.*, 1990; and Zheng *et al.*, 1990). EPL can affect membrane properties such as permeability (Bittman *et al.*, 1984) and fluidity (Paltauf, 1994). EPL influence signal transduction to many metabolic pathways by protein kinase C (PKC) (Zheng *et al.*, 1990), Na-K-ATPase (Oishi *et al.*, 1988), inositol-lipid turnover (Seewald *et al.*, 1990), and intracellular calcium (Alonso *et al.*, 1997). EPL induce cell apoptosis (Alonso *et al.*, 1997), cytotoxicity (Diomede *et al.*, 1993; Honma *et al.*, 1981), and antitumor activity (Mavromoustakos *et al.*, 2001; Principe *et al.*, 1994), which could have potential in anti-cancer applications. Selective cytotoxic effects of EPL is dependent on membrane cholesterol amount (Diomede *et al.*, 1990). For example, HL60 cells with a high cholesterol content show lower uptake of EPL into membranes, resulting in decrease in membrane fluidity (Diomede *et al.*, 1990) and higher rates of apoptosis (Diomede *et al.*, 1993). Alkyl-lysophospholipids exhibit strong selective cytotoxicity in leukemia cells but not in normal bone marrow cells (Honma *et al.*, 1981).

[0007] Gangliosides and EPL may perform similar functions. For example, both of these types of lipids localize in neuronal (Byrne *et al.*, 1983; Svennerholm *et al.*, 1989; and Horrocks 1972) and intestinal tissues (Forstner *et al.*, 1973; Paltauf, 1972). Both gangliosides and EPL exhibit anti-cancer effects (Mavromoustakos *et al.*, 2001; Principe *et al.*, 1994; Schmelz *et al.*, 2000) and contribute to cell differentiation (Ledeen *et al.*,

1998; Honma *et al.*, 1981) and apoptosis (Diomede *et al.*, 1993; Malisan *et al.*, 2002). Gangliosides and EPL are sensitive to membrane cholesterol content (Diomede *et al.*, 1990; Blank *et al.*, 1992).

[0008] In American diets daily intake of SPL (including gangliosides and sphingomyelin) is about 300 mg (Vesper *et al.*, 1999) and daily intake of EPL is about 1 mg per gram of food (Berger *et al.*, 2000). Neonates consume SPL and EPL from mothers milk (Diomede *et al.*, 1991), but the metabolic interaction between dietary SPL and EPL is not known.

[0009] Cholesterol reduction in membranes causes increased EPL uptake (Diomede *et al.*,1990; Leikin *et al.*, 1988) and increased activity of Δ-5 and Δ -6 desaturase enzymes (Clandinin *et al.*, 1991). Dietary gangliosides may increase total membrane EPL content and accompanied with higher polyunsaturated fatty acid (PUFA) in subclasses of EPL. Sphingomyelin can be used as a control to compare bioavailability with gangliosides because sphingomyelin and gangliosides have the same ceramide molecule anchored in the cell membrane, but attached to a different head group. Using rats, the present data illustrated herein demonstrates that dietary ganglioside increases total content and composition of EPL containing PUFA in the developing intestine.

[0010] ***Microdomains.*** Microdomains, generally called lipid rafts, caveolae, or glycosphingolipid-signaling domains, have been characterized as important domains for signal transduction and lipid (i.e. cholesterol) and protein trafficking (Anderson, 1998; Brown *et al.*, 1998; Hakomori *et al.*, 2000; and Simons *et al.* 1997). Microdomains are recently known as a site for the cellular entry of bacterial and viral pathogens (Fantini, 2000; Katagiri*et al.*, 1999; and Bavari *et al.*, 2002). For instance, the entry of filoviruses requires lipid rafts as the site of virus attack (Bavari *et al.*, 2002). Cholera toxin entered the cell by endocytosis GM1 as the sorting motif necessary for retrograde trafficking into host cells and such trafficking depends on association with lipid rafts (Wolf *et al.*, 2002).

[0011] Physiological and functional roles of microdomains are dependent on cholesterol and sphingolipids including gangliosides. Reduction of cholesterol inhibits pathogen entry by disrupting the structure of microdomains (Popik *et al.*, 2002; Samuel *et al.*, 2001) and impairs inflammatory signalling (Wolf *et al.*, 2002; Triantafilou *et al.*, 2002). Cholesterol upregulates the expression of caveolin, a marker of protein for caveolae (Fielding *et al.*,

1997; Hailstones *et al.*, 1998). Sphingolipid depletion inhibits the intracellular trafficking of GPI-anchored proteins and endocytosis via GPI-anchored proteins (Kasahara *et al.*, 1999), suggesting that lipid-protein interaction directly modulates gene expression and cellular trafficking important for cell development and behaviour.

[0012] The neonatal intestine has permeable, endocytic and enzymatic transport systems for absorption of nutrients and immunoglobulins (Moxey *et al.*,1979; Wilson *et al.*, 1991) but is susceptible to pathogen entry because of higher permeability than that of adults (Koldovsky 1994). High amount of gangliosides in mothers' milk during the neonatal period therefore act as a receptor for viral and bacterial toxins to protect entry of pathogens into enterocytes (Rueda *et al.*, 1998). During development, membrane permeability gradually decreases (Koldovsky 1994) while peptidases and glycosidases become functionally active and enriched in microdomains (Danielsen *et al.*, 1995). Many digestive/absorptive enzymes, such as alkaline phosphatase, aminopeptidase N and A, and sucrase-isomaltase are also increased in apical membrane microdomains (Stulnig *et al.*, 2001). These results seem to suggest the importance of microdomains of intestinal apical membranes for nutrient uptake and metabolism.

[0013] Polyunsaturated fatty acids (20:5n-3 or 22:6n-3) can accumulate in microdomains and displace functional proteins by changing the lipid composition of the microdomain (Stulnig *et al.*, 2001; Williams *et al.*, 1999). This observation highlights the importance of dietary lipids in modulating physiological and biological properties of proteins in the microdomain. Little is known of how dietary gangliosides affect the lipid profile and protein components of microdomains during neonatal gut development.

[0014] Some previous studies have suggested that cholesterol depletion inhibits inflammatory signaling by disrupting microdomains structure (Wolf *et al.*, 2002; Samuel *et al.*, 2001; Triantafilou *et al.*, 2002). However, it has not been evaluated whether diet-induced cholesterol reduction has any effect on decreasing cholesterol in the microdomain, disrupting microdomain structure and reducing pro-inflammatory mediators such as diglyceride (DG) and platelet activating factor (PAF). DG derived from phospholipids by phospholipase C, binds to protein kinase C (PKC) to phosphorylate targeted proteins, such as the epidermal growth factor receptor and DG resides in microdomains (Sciorra *et al.*, 1999; Smart *et al.*, 1995). The instant invention assesses these effects.

[0015] PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine, stimulates inflammatory cells such as leukocytes (Prescott *et al.*, 1990) and activates phospholipase A2 (PLA2) in the intestinal tissue to release arachidonic acid (Okayasu *et al.*, 1987). Meanwhile, increased lyso-PC by PLA2 is further used for PAF synthesis with an acetylcholine transferase. PAF binds its receptor to increase intracellular calcium and inositol triphosphate (IP3) production and PKC activation for inflammation (Flickinger *et al.*, 1999). It is unknown if PAF also localizes in the microdomain. Since several studies reported that sphingomyelin (SM), a sphingolipid, has an inhibitory effect on PLA2 activity (Koumanov *et al.*, 1997), it was of interest to determine if dietary ganglioside also decreases PAF synthesis either by increasing sphingolipids or by disrupting microdomains structure in developing intestine. We also examine if dietary ganglioside reduces DG content in the microdomain since sphingosine, a derivative of sphingolipids inhibits PKC signaling which is required a structural complex with DG.

[0016] Neonates consume SPL including gangliosides from mothers milk (Carlson 1985; Berger *et al.*, 2000). Gangliosides are known to act as receptors for viruses and toxins (Laegreid *et al.*, 1987; Rolsma *et al.*, 1998), activators for T-cells (Ortaldo *et al.*, 1996) and stimulators for Th-1 and Th-2 cytokine-secreting lymphocytes in neonates (Vazquez *et al.*, 2001). Gangliosides are also one of the major lipid components in microdomains. It is not known if dietary ganglioside changes the lipid profile and structure of the intestinal microdomain and modulating inflammatory signalling mechanisms in the developing intestine. Thus the objective of the present study was to determine if dietary ganglioside increases gangliosides and decreases cholesterol and caveolin content in the intestinal microdomain leading to disruption of microdomain structure and anti-inflammatory signals in the developing gut.

[0017] It is desirable to find a compound, a class of compounds, or composition active in mediating inflammation. It is also desirable to find such compounds or compositions that is naturally occurring in the food supply, so as to more easily meet with public acceptance.

[0018] Further, it is desirable to find a compound, a class of compounds, or composition active in mediating inflammation. Advantageously, such compounds or compositions

would be naturally occurring in the food supply, so as to more easily meet with public acceptance.

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[00216] ***Abbreviations used herein are as follows:***

[00217] CPG: Choline phosphoglycerides; DG: diacylglycerol; E. Coli: *Escherichia coli*; EPG: Ethanolamine phosphoglycerides; EPL: Ether phospholipids; Gang-High: High concentration of ganglioside; Gang-Low: Low concentration of ganglioside; GD1b: II3 $(NeuAc)_2$-$GgOse_4Cer$; GD3: Ganglioside GD3 :II^3 $(NeuAc)_2$-LacCer; GLC: Gas liquid chromatography; GG: gangliosides; GM1: Ganglioside GM1; II^3 NeuAc-$GgOse_4Cer$; GM2: Ganglioside GM2: II^3 NeuAc-$GgOse_3Cer$; GM3: Ganglioside GM3: II^3 NeuAc-LacCer; LCPUFA: Long chain polyunsaturated fatty acids; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; MUFA: monounsaturated fatty acids; NANA: N-Acetyl neuraminic acid; PAT: platelet activating factor; PBS: Phosphate buffered saline solution; PC: phosphatidylcholine; PE: phosphatidyl ethanoloamine; PI: phosphatidylinositol; PKC: Protein kinase C; PL, phospholipids; PS: phosphatidylserine; PUFA: polyunsaturated fatty acids; SEM: Standard error of the mean; SFA: Saturated fatty acids; sIgA: Secretory immunoglobulin A; SM: sphingomyelin; SPL: Sphingolipids; TG: Triglyceride; and TLC: Thin layer chromatography.

SUMMARY OF THE INVENTION

[00218] It is an object of the present invention to provide a formulation, including a compound, a class of compounds or a composition active in the mediation of inflammation, particularly inflammation of the intestinal, retinal or neural tissue. Such mediation of inflammation can decrease cholesterol absorption.

[00219] According to embodiments of the invention, a formulation is provided that is effective in mediating inflammation, including inflammation of the intestine, retina or neuronal tissue. Further, according to an embodiment of the invention, a formulation is provided for reducing blood cholesterol level. The formulations according to the invention may include a ganglioside such as GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid.

[00220] Embodiments of the invention also relate to a method for mediating inflammation and a method for reducing blood cholesterol in a subject in need thereof, comprising the step of providing at least one ganglioside to the subject for oral consumption. Uses of at least one ganglioside for preparation of a medicament for oral

consumption to mediate inflammation and to reduce blood cholesterol in a subject in need thereof are also encompassed by the invention.

[00221] Other aspects and features of the present invention will become apparent to those ordinarily skilled in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[00222] Embodiments of the present invention will now be described, by way of example only, with reference to the attached Figures, wherein:

[00223] **Figure 1** illustrates the ratio of SFA to MUFA (white columns) and SFA to PUFA (black columns) in alkenylacyl-, alkylacyl- and diacyl subclasses in CPG in intestinal mucosa of animals fed control or treatment diets.

[00224] **Figure 2** illustrates the ratio of SFA to MUFA (white columns) and SFA to PUFA (black columns) in alkenylacyl-, alkylacyl- and diacyl subclasses in EPG in intestinal mucosa of animals fed control or treatment diets.

[00225] **Figure 3** shows the fatty acid composition of alkenylacyl, alkylacyl and diacyl subclasses in CPG in intestinal mucosa of animals fed control diet or treatment diets.

[00226] **Figure 4** shows the fatty acid composition of alkenylacyl, alkylacyl and diacyl subclasses in EPG in intestinal mucosa of animals fed control diet or treatment diets.

[00227] **Figure 5** shows total content of GG (A), SM (B) and cholesterol (C) in intestinal microdomains after feeding different diets for 2 wks.

[00228] **Figure 6** illustrates total content of GD3 (A) and PAF (B) in intestinal microdomains after feeding different diets for 2 wks.

[00229] **Figure 7** shows the ratio of cholesterol/GG (A) and cholesterol/SM (B) in intestinal microdomains after feeding different diets for 2 wks.

[00230] **Figure 8** shows caveolin content determined by western blotting (A), and the intensity of blots (B) in intestinal microdomains fed control diet or treatment diets for 2 wks.

[00231] **Figure 9** illustrates the total content of a) gangliosides and b) phospholipids in the retina of control and treatment groups.

[00232] **Figure 10** illustrates immunofluorescent detection of GM3 localization.

[00233] **Figure 11** illustrates immunofluorescent detection of GD3 localization.

[00234] **Figure 12** shows the effect of dietary treatment on total content of gangliosides in (A) the intestinal mucosa, (B) plasma and (C) brain for animals fed either the control or experimental diet for two weeks.

[00235] **Figure 13** illustrates the effect of dietary treatment on cholesterol content in (A) the intestinal mucosa, (B) plasma and (C) brain of animals fed either the control or experimental diets for two weeks.

[00236] **Figure 14** shows the effects of dietary treatment on the ratio of cholesterol to ganglioside in (A) the intestinal mucosa, (B) plasma and (C) brain of animals fed either the control or experimental diets for two weeks.

[00237] **Figure 15** illustrates the composition of GM3 and GD3 in microdomains of rat intestine.

[00238] **Figure 16** illustrates the composition of PAF and DG in microdomains of rat intestine. Both PAT and DG are reduced with a GG diet.

[00239] **Figure 17** illustrates the caveolin content of microdomains for animals fed a control, PUFA or GG diet.

[00240] **Figure 18** illustrates that feeding a diet high in ganglioside resulted in decreased plasma cholesterol and triglyceride.

DETAILED DESCRIPTION

[00241] Generally, the present invention provides a formulation for mediating inflammation, for example, inflammation of the intestine, of the retina, or of neural tissue other than the retina. Further, a formulation is provided for lowering blood cholesterol.

[00242] The invention is based in the discovery of a ganglioside containing composition, such as a milk derived dietary component, that alters inflammation mediators and has an effect in cholesterol lowering. Inflammation mediation occurs particularly in the intestine, the retina or other neural tissue. The invention is also particularly useful in treating or preventing inflammatory diseases and in reducing blood cholesterol levels, possibly through decreased intestinal absorption of cholesterol.

[00243] According to embodiments of the invention, a formulation is provided that is effective in mediating inflammation. Inflammatory states to be mediated include

inflammation of the intestine, retina or neuronal tissue, and by "mediating" it is meant preventing or treating an inflammatory disease. For example, such diseases may include inflammatory bowel disorders, disorders arising from allergic responses, and diseases involving epithelial surface responses. Gastroenteritis, enteric infections, enterocolitis, and necrotizing enterocolitis are all examples of such inflammatory conditions that may be alleviated according to the invention. Infants may be particularly susceptible to such conditions.

[00244] According to an embodiment of the invention, a formulation is provided for reducing plasma cholesterol level.

[00245] The formulations according to the invention may include a ganglioside such as GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid. The formulation may be in the form of a supplemented liquid or food, such as infant formula or infant foods. An exemplary formulation may a total ganglioside composition made up of the following individual components: about 80% GD3, 9% GD1b, and 5% GM3 on a weight/weight basis. A further exemplary formulation may have one predominant ganglioside, for example: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid, that comprises more than 50% of the total ganglioside content.

[00246] An embodiment of the invention also relates to a method for mediating inflammation in a subject in need thereof comprising the step of providing at least one ganglioside to the subject for oral consumption. The use of at least one ganglioside for preparation of a medicament for oral consumption to mediate inflammation in a subject in need thereof is also encompassed by an embodiment of the invention.

[00247] Further, an embodiment of the invention encompasses a method for reducing blood cholesterol in a subject in need thereof comprising the step of providing at least one ganglioside to the subject for oral consumption. Additionally, the invention provides for the use of at least one ganglioside for preparation of a medicament for oral consumption to reduce blood cholesterol in a subject in need thereof.

[00248] Experiments were done to assess the effects of dietary gangliosides, for example: components isolated from milk, on a variety of parameters indicative of or causal in mediating an inflammatory or anti-inflammatory response. These experiments lead to the identification of gangliosides responsible for prophylactic and/or therapeutic effects.

[00249] According to the invention, a ganglioside fraction, for example a fraction derived from milk, herein referred to as "Fraction A" may be used. Other sources of gangliosides, such as from dairy products or synthetic sources, may also be used in preparing the formulations according to the invention.

[00250] The dosage amount of the ganglioside formulation according to the invention that may be used for oral delivery can easily be determined by one of skill in the art. A daily or one-time only minimum dosage may be from microgram to milligram quantities. A higher level may have a greater effect where the exposure and likelihood of infection is increased. A formulation in food or fluid form having from 1 to 1000 ppm of one or more ganglioside can be delivered to a subject in need thereof. A concentration falling outside of this range may also be used, and no upper limit is required because the formulation does not display toxicity, and is not know to be toxic.

[00251] To accomplish an inflammation mediating effect or a cholesterol lowering effect, a typical dosage for adults may be from about 100 mg to about 1 g of ganglioside per person per day, based on an adult body weight of about 70 kg. However, it is possible to deliver gangliosides in a quantity outside of this range, for example from about 10 mg to about 10 g may be effectively delivered to an individual in need thereof.

[00252] For an infant having a typical body weight of about 3.5 kg, a level of gangliosides that may be delivered in order to accomplish an inflammation mediating effect may range from about 10 to about 50 mg per day per infant. However, it is possible to deliver gangliosides in a quantity outside of this range, for example from about 1 mg to about 100 mg may be delivered in order to accomplish an inflammation mediating effect.

[00253] Fraction A may be prepared by crude processes, or may be obtained commercially from a source such as New Zealand Dairy, New Zealand. Fraction A is of variable lipid composition, for example, approximately 80% GD3; 9% GD1b, and 5% GM3 by weight, the remaining 6% being comprised of other gangliosides. Fraction B, higher in GM3 can also be prepared depending on the milk used for ganglioside isolation.

[00254] Other molecular forms of this complex lipid may be derived, according to the invention, with similar or even greater bioactive characteristics. According to one possible composition of the formulation used in the instant invention, the fraction may contain one

or more gangliosides, such as for example GD3, GM1, GM2, GM3, GD1b, NANA; and sialic acid and is bioactive against *Giardia* producing very high kill rates.

[00255] Certain components of the formulation of the invention can be isolated from components of the present food supply, and thus would not need "drug" approval to be added to or to enriched new foods.

[00256] Ganglioside supplementation, or supplementation of a lipid fraction containing ganglioside can be used to supplement or fortify existing foods, such as in infant formulas, baby foods, baby cereals, and follow-on formulas which may be used for children up to about 18 months of age. Further, supplementation may also be useful in juices or other fluids packaged particularly for toddlers or older children, or in cereals as a coating or powdered sprinkle. Such foods may advantageously be those which are appealing to children, as this could be used to treat or prevent inflammatory disease at an early age, or to mediate inflammation.

[00257] Foods appealing to adults may also be supplemented or fortified with gangliosides or ganglioside-containing fractions in order to target individuals requiring mediation of an inflammatory response. The use of such foods, in treating or preventing inflammatory diseases or to lower blood cholesterol level, is also encompassed by the present invention.

[00258] In addition to being supplemented into food, the formulation may be provided in a liquid, gel, powder, tablet, pill or capsule form. Tablet, pill or capsule form may appeal to older children and adults, and would avoid the need to consume a food or beverage.

[00259] The supplement may also be added to pet foods or supplements, or to foods directed to other domesticated animals. In some instances, it may also be desirable to supplement the formulation to livestock. Such a use of gangliosides would be helpful in mediating an inflammatory response in animals in need thereof.

[00260] Example 1

[00261] ***Milk Fraction A Containing Gangliosides***

[00262] **Table 1** provides the composition of Fraction A, illustrating the amount of total lipid, calcium and lactose present in 100g of Fraction A on a dry weight basis. The ganglioside and phospholipid content of the lipid fraction is broken down into specific

components. In **Table 1**, all abbreviations used are those defined previously, and additionally: %GG means percent of total gangliosides; %PL means percent of total phospholipids; x-1, x-2 and x-3 are gangliosides; LPC: lysophosphatidylcholine; SM: sphingomyelin; PC: phosphatidylcholine; LPE: lysophosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol; PE: phosphatidyl ethanoloamine.

Table 1
Composition of Fraction A

Fraction A	**100g**		
Total Lipids(g)	23.00		
		(g)	
Gangliosides (as NANA amt)		0.82	(% GG)
	GM3		4.50
	x-1		4.60
	x-2		0.80
	GD3		79.90
	GD1b		9.00
	x-3		1.20
		(g)	
PL (as 'P')		0.49	(% PL)
	LPC	0.036	7.3
	SM	0.013	2.7
	PC	0.012	2.5
	LPE	0.093	19.0
	PS	0.149	30.4
	PI	0.136	27.8
	PE	0.050	10.2
		0.49	99.9
Neutral lipid	0.04		
Cholesterol	0.08		
Ca(g)	10.00		
Lactose(g)	65-70		

[00263] Example 2

[00264] ***Separation of Gangliosides from Fraction A***

[00265] Fraction A, having the composition described above in **Table 1, Example 1,** was obtained and gangliosides were separated therefrom using the following method. The separated ganglioside fraction so obtained may be used in a supplementation regime according to the invention. Alternatively, individual gangliosides obtained from the separated fraction may be used in a supplementation regime according to the invention.

[00266] Total lipids were extracted from the ganglioside enriched preparation of Fraction A using the Folch method (Folch and Sloane-Stanley, 1957). The ganglioside-containing upper phase was transferred and the lower phase was washed once with Folch upper phase solution (chloroform/methanol/water, 3/48/47 by vol.). The combined ganglioside-containing fractions were passed through Sep-Pak™ C18 reverse-phase cartridges (Waters Corporation, Milford, MA, USA), eluted with methanol and chloroform and methanol 2:1 (v/v), and dried completely under vacuum at 23°C using a rotary evaporator. Ganglioside (NANA) content was measured as described by Suzuki (1964).

[00267] **Example 3**

[00268] ***Dietary Gangliosides Increase Content of Ether Phospholipids Containing 20:4n-6 and 22:6n-3 in the Rat Intestine***

[00269] In this Example, the effect of dietary gangliosides on the content of ether phospholipids (EPL) in intestinal mucosa was observed. Male Sprague-Dawley rats (18-day old) were fed a semi-purified diet consisting of 20% fat as a control diet. Two experimental diets were formulated by adding either 0.1% (w/w) gangliosides (GG diet) or 0.2% (w/w) sphingomyelin (SM diet) to the control diet. Fatty acid methyl esters from the alkenylacyl, alkylacyl, and diacyl subclasses of phospholipids were measured to determine total and relative percentage of EPL comprising the choline phosphoglyceride (CPG) and ethanolamine phosphoglyceride (EPG) fractions. The GG diet was shown to increase the overall levels of EPL in both CPG and EPG in intestinal mucosa as well as increasing the PUFA content of the EPL class, specifically in 20:4n-6 and 22:6n-3. As a result of the increase in PUFA content, the ratio of SFA to PUFA in both CPG and EPG was reduced in animals fed the GG diet. The effects of the SM diet were similar but of lesser magnitude than the GG diet. Enhanced EPL content may suppress carcinogenesis, inflammation and lipid oxidative processes in the developing intestine.

[00270] ***Materials and Methods***

[00271] ***Animals and diets.*** The protocol for this study was approved by the Animal Care Committee at the University of Alberta, Canada. Male Sprague-Dawley rats (18-day old, 40 ± 4.5g) were housed in polypropylene cages and maintained at a constant room temperature of 23°C and a 12 h light/dark cycle for 2 weeks. Animals had free access to

water and were randomized to be fed one of three semi-purified diets containing 20% (w/w) fat (**Table 2**). The composition of basal diet is reported elsewhere (Clandinin *et al.*, 1980). The fat in the control diet was a blend of triglycerides reflecting the overall fat composition of an infant formula. Two experimental diets were formulated by adding either sphingomyelin (0.2% w/w, Sigma, MO, USA; SM diet) or a ganglioside enriched lipid powder (0.1% w/w, New Zealand Dairy, New Zealand; GG diet) to the control diet. The ganglioside enriched lipid powder consisted of about 45-50% (w/w of total lipid) as phospholipid and 15-20% (w/w of total lipid) as gangliosides. The ganglioside fraction contained about 80% (w/w) GD3 and GD1b, GM3 and other gangliosides (9, 5 and 6% w/w, respectively). The fatty acid composition of experimental diets was quantitatively analyzed by gas liquid chromatography (GLC, Varian Vista 3400CX). The fatty acid composition was consistent among the three diets: 18:1n-9 (50%), 18:2n-6 (20%), 16:0 (16%), 18:0 (8%), 18:3n-3 (2.8%) and other fatty acids (3.2%). The control diet and experimental diets provided an n-6 to n-3 ratio of 7.1. The cholesterol amount was low (<0.35%, w/w of total lipid). Overall the GG diet contained 19.6% triglycerides, 0.1% gangliosides, 0.2% phospholipid and 0.07% cholesterol (**Table 2**). Body weight and food intake was measured every other day throughout the experiment.

Table 2
Composition of experimental diets[1]

	Control	SM	GG
Basal diet (g/100g)	80.0	80.0	80.0
Triglyceride	20.0	19.8	19.6
Sphingomyelin	-	0.2	tr[2]
Ganglioside	-	-	0.1
Phospholipid	-	-	0.2
Cholesterol	-	-	tr

[1] The composition of the control diet are referred from Clandinin *et al.*, 1980, representing an existing infant formula.
[2] tr presents trace amount.

[00272] ***Collection of intestinal mucosa.*** After anesthetizing animals with halothane, the small intestine (jejunum to ileum) was excised. The intestine was washed with 0.9 % cold saline solution to remove visible mucus and debris, opened, and moisture was carefully removed. Intestinal mucosa was scraped off with a glass slide on an ice cold glass plate. All samples were weighed and kept in a –70°C freezer until used.

[00273] ***Lipid extraction and phospholipid separation.*** Total lipid was extracted by using the Folch method (Folch *et al.*, 1957). For extracting total lipid, the intestinal mucosa was washed twice with Folch lower phase solution (chloroform/methanol/ water, 86:14:1, v/v/v). The lower phase lipid was pooled, dried and then dissolved in chloroform:methanol (2:1, v/v). Extracted lipid was applied onto pre-coated silica gel "H" thin layer chromatography (TLC) plates (Analtech, Newark, DE) and developed in the solvent (chloroform/methanol/2-propanol/0.25% KCl/triethylamine, 45:13.5:37.5:9:27, v/v/v/v/v) to separate individual phospholipid classes. After spraying with 0.1 % ANSA (anilino naphthalene sulfonic acid) and identifying CPG and EPG bands under UV light, the two bands were scraped into test tubes.

[00274] ***Fatty acid composition and quantification.*** Solid phases containing CPG and EPG were eluted with 5 mL of chloroform:methanol (2:1) and then dried under N_2. The dried phospholipids were dephosphorylated with phospholipase C (B. cereus, Sigma Chemical Co., St. Louis, MO) at 37°C for 2 hr in a solution of 1 mL diethyl ether, 4 mL 17.5 mM Tris buffer (pH 7.3), and 1.3 mL 1% $CaCl_2$ (Bernett *et al.*, 1985). After extraction of the hydrolyzed lipids with diethyl ether and petroleum ether, lipids were acetylated in 0.1 mL of pyridine with 0.5 mL of acetic anhydride at 80 °C for 1 hr. The acetylated derivatives of alkenylacyl, alkylacyl and diacyl phospholipids were extracted with chloroform/methanol (2:1) solution , applied onto a silica gel high performance TLC plate (HPTLC, Whatman Inc, Clifton, NJ) and developed in petroleum ether: diethyl ether: acetic acid, (90:10:1, v/v/v) to a migration distance of 10 cm from the solvent line, followed by a second development in toluene (Holub *et al.*, 1987). The plate was sprayed with ANSA and visualized under UV light. Three subclasses of the CPG and EPG fractions scraped from the plate were then methylated in 3N-methanolic-HCl (Supelco, PA, USA) for 16 hr at 70°C with a known amount of heptadecanoic acid as an internal standard. The fatty acid composition of each of the six fractions was analyzed by GLC

equipped with a flame ionization detector and BP-20 fused capillary column (SGE, Australia). The flow rate of helium gas was 1.6 mL/min and the oven, injector and detector temperatures were 200°C, 250°C, and 250°C, respectively. Since only acylated fatty acids (not ethers) can be converted to fatty acid methyl esters for GLC analysis, the fatty acid amount measured in EPL only represents one-half of the total. Thus, this value was multiplied by two to have total and molecular percentage of EPL.

[00275] ***Statistical analysis.*** The values were shown as means ± standard deviation from eight animals, with a few exceptions indicated. Significant differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with the SAS system. Effects of diet treatment were determined by a Duncan multiple range test at significance levels of $P < 0.05$, $P < 0.01$, $P < 0.001$ or $P < 0.0001$.

[00276] ***Results***

[00277] ***Animal growth and intestinal mucosa.*** Initial body weight of animals, weight after two weeks of diet treatment was not significantly different between experimental and control groups. Intestinal mucosal weight and length was not affected by dietary treatment. Food consumption was also not influenced by diet treatment.

[00278] ***Fatty acid content corresponding to alkenylacyl, alkylacyl and diacyl phospholipids in CPG and EPG.*** Animals fed the GG diet presented significantly higher amount of fatty acids from alkenylacyl-CPG, alkenylacyl-EPG and alkylacyl-EPG in comparison with control animals (56%, 77% and 54% increases, respectively; **Table 3**). The highest change in fatty acid content relative to the control was observed in alkenylacyl-EPG ($P<0.0008$). In animals fed dietary gangliosides, a significant decrease in diacyl-CPG occurred, whereas no change was observed in diacyl-EPG. Animals fed the SM diet exhibited a similar increase in fatty acid content corresponding to alkylacyl-GPE, but no change occurred in the other subclasses of GPC and GPE. In the total fatty acid content of EPL (alkenylacyl and alkylacyl together), the GG diet produced significant increase, by 36% and 66% respectively, comprising CPG and EPG phospholipids in intestinal mucosa (**Table 3**). Feeding animals the SM diet only increased total EPL in EPG by 42% compared to control animals.

Table 3
Fatty acid content of alkenylacyl, alkylacyl and diacyl subclasses in CPG and EPG from intestinal mucosa of animals fed experimental diets[1]

Subclass (μg/g tissue)	Control	SM	GG	Effect of diet (P<)
Total EPL-CPG[2]	31.8 ± 6.7 [b]	37.8 ± 8.7 [ab]	43.3 ± 8.2 [a]	0.05
Alkenylacyl-CPG	14.0 ± 3.6 [b]	17.0 ± 3.0 [ab]	21.8 ± 7.2 [a]	0.05
Alkylacyl-CPG	17.8 ± 3.9	20.8 ± 7.5	21.5 ± 4.8	
Diacyl-CPG	1.86×10^3 ± 344 [a]	1.61×10^3 ± 469 [ab]	1.22×10^3 ± 306 [b]	0.01
Total EPL-EPG	68.1 ± 7.9 [c]	97.1 ± 15.3 [b]	113 ± 20 [a]	0.001
Alkenylacyl-EPG	34.4 ± 7.7 [b]	45.2 ± 14.5 [b]	60.9 ± 12.3 [a]	0.0008
Alkylacyl-EPG	33.7 ± 4.4 [b]	51.9 ± 10.6 [a]	51.8 ± 10.7 [a]	0.0006
Diacyl-EPG	774 ± 99	731 ± 292	801 ± 174	

[1] Mean ± SD from 7, 8, and 7 animals, for the Control, SM and GG group, respectively.
[2] Fatty acid content of total EPL (alkenylacyl and alkylacyl together) in CPG or EPG.

[00279] ***Changes in relative amounts of SFA, MUFA, and PUFA in alkenylacyl, alkylacyl and diacyl phospholipids comprising CPG and EPG classes.*** As PUFA levels in alkylacyl-CPG increased in animals fed the GG diet, there was a concomitant decrease in the ratio of SFA to PUFA in alkylacyl-CPG (2.6 versus 6.8, P<0.05; **Figure 1**). In contrast, in diacyl-CPG fractions, animals fed the GG diet exhibited an increase in the ratios of SFA to MUFA (5.3 versus 3.6, P<0.0007) and SFA to PUFA (1.5 versus 1.1, P<0.05).

[00280] **Figure 1** illustrates the ratio of SFA to MUFA (white columns) and SFA to PUFA (black columns) in alkenylacyl-, alkylacyl- and diacyl subclasses in CPG in intestinal mucosa of animals fed control or treatment diets. Values are means ± SD of 7, 8 and 7 animals for the control, SM and GG diet, respectively. Letters represent a significant difference between groups at P<0.05, except for the ratio of SFA to MUFA in the diacyl subclass at P<0.0007.

[00281] Changes in relative amounts of SFA, MUFA, and PUFA in the three subclasses of EPG are illustrated (**Figure 2**). In the GG diet group, alkenylacyl-EPG and alkylacyl-EPG exhibited significantly lower ratios of SFA to PUFA compared to the controls (0.6 versus 1.3 for alkenylacyl-GPE, $P<0.005$, and 0.5 versus 1.2 for alkylacyl-GPE, $P<0.05$). There was also a decrease in the SFA/MUFA ratio (1.8 versus 2.5) in the alkylacyl group. Animals fed the SM diet exhibited a lower SFA/PUFA in the both EPL subclasses compared to animals fed the control diet, but effects were more subtle than that of the GG diet. There was no observed effect of SM or GG treatments on the diacyl-EPG class.

[00282] **Figure 2** shows the Ratio of SFA to MUFA (white columns) and SFA to PUFA (black columns) in alkenylacyl-, alkylacyl- and diacyl subclasses in EPG in intestinal mucosa of animals fed control or treatment diets. Values are means ± SD of 7, 8 and 7 animals for the control, SM and GG diet, respectively. Letters represent a significant difference between groups at $P<0.005$, except for the ratio of SFA to PUFA in the alkylacyl subclass at $P<0.02$.

[00283] ***Changes in relative composition of alkenylacyl, alkylacyl and diacyl phospholipids comprising CPG and EPG.*** Animals fed the GG diet exhibited higher levels of alkenylacyl-CPG (3.3% versus 1.6%) and alkylacyl-CPG (3.2% versus 2.1%) with correspondingly lower levels of diacyl-CPG (93.5% versus 96.3%), compared to animals fed the control diet (**Table 4**). No effect of the SM diet was observed on the relative composition of CPG in the mucosa. Feeding animals the GG diet achieved higher levels of alkenylacyl-EPG (12.5% versus 7.4%) and alkylacyl-EPG (10.9% versus 7.3%) with correspondingly lower levels of diacyl-EPG (76.6% versus 85.3%), compared to animals fed the control diet. Animals fed the SM diet showed a relative increase in alkenylacyl-EPG, but the effect was less pronounced than in those fed the GG diet. There was no effect of the SM diet on alkylacyl-EPG. Relative to total phospholipids, animals fed the GG di*et also* exhibited a marked increase in total EPL (alkenylacyl and alkylacyl together) from 3.7% to 6.5% in CPG and from 14.7% to 23.4% in EPG (**Table 4**). Feeding the SM diet resulted in a relative increase in EPG, but not in CPG.

Table 4
Percentage of alkenylacyl, alkylacyl and diacyl subclasses in CPG and EPG from intestinal mucosa of animals fed experimental diets[1]

Subclass (%, w/w)	Control	SM	GG	Effect of diet (P<)
Total-EPL-CPG[2]	3.7 ±0.8 [b]	4.8 ± 1.1 [b]	6.5 ± 1.8 [a]	0.006
Alkenylacyl-CPG	1.6 ±0.4 [b]	2.2 ± 0.8 [ab]	3.3 ± 1.3 [a]	0.05
Alkylacyl-CPG	2.1 ±0.5 [b]	2.6 ± 0.6 [ab]	3.2 ± 0.8 [a]	0.05
Diacyl-CPG	96.3 ±0.8 [a]	95.2 ± 1.1 [a]	93.5 ± 1.8 [b]	0.007
Total-EPL-EPG	14.7 ±1.6 [b]	20.4 ± 5.4 [a]	23.4 ± 3.2 [a]	0.001
Alkenylacyl-EPG	7.4 ±1.5 [c]	10.2 ± 2.1 [b]	12.5 ± 1.4 [a]	0.0001
Alkylacyl-EPG	7.3 ±1.1 [b]	10.2 ± 3.7 [ab]	10.9 ± 2.5 [a]	0.05
Diacyl-EPG	85.3 ±1.6 [a]	79.6 ± 5.4 [b]	76.6 ± 3.2 [b]	0.002

[1] Mean ± SD from 7, 8, and 7 animals, for the Control, SM and GG group, respectively. [2] Percent of total EPL (alkenylacyl and alkylacyl together) relative to total phospholipids in CPG or EPG. Changes in fatty acid composition of alkenylacyl-, alkylacyl- and diacyl-CPG

[00284] ***Changes in fatty acid composition of alkenylacyl-, alkylacyl- and diacyl-CPG.*** In comparison with control animals, animals fed the GG diet did not show significant change in the fatty acid composition of alkenylacyl-CPG except for 14:1, which decreased (**Figure 3**). Animals fed the GG diet showed an increase in 20:4n-6 as compared to animals fed the SM diet. Decrease occurred in alkylacyl-CPG content of 18:0, 24:0 and 24:1 for animals fed the GG diet as well as a distinct increase in 22:6n-3 ($P<0.0007$). The SM diet produced a decrease in 18:0 and an increase in 22:6n-3 content. In diacyl-CPG, higher levels of 16:0 and 18:0 and lower levels of 18:1n-9 and 18:2n-6 were observed in animals fed the GG diet compared to control animals. Similar trends in 16:0, 18:1n-9 and 18:2n-6 were observed for animals fed the SM diet.

[00285] ***Changes in fatty acid composition of alkenylacyl-, alkylacyl- and diacyl-EPG.*** The fatty acid composition of alkenylacyl-, alkylacyl, and diacyl-EPG in intestinal mucosa of animals fed experimental diets is illustrated (**Figure 4**). Animals fed dietary

gangliosides showed higher levels in alkenylacyl-EPG of 22:4n-6 (100% increase, P<0.001) and 22:6n-3 (71% increase, P<0.001) compared to controls, and lower levels of 16:0 and 18:0. Animals receiving the SM diet exhibited a similar change in 22:4n-6, 22:6n-3 and 16:0 fatty acid content, but the effect was smaller than observed for animals fed the GG diet. In alkylacyl-EPG, higher content of 20:4n-6, 22:4n-6, and 22:6n-3 (36%, 87% and 77% increases, respectively) was observed in animals fed the GG diet with a considerable reduction of saturated fatty acids 16:0, 18:0 and 24:0 relative to animals fed the control diet. In diacyl-EPG, dietary gangliosides increased the content of 20:4n-6 by 36%, relative to controls.

[00286] ***Changes in total SFA, MUFA and PUFA content of alkenylacyl-, alkylacyl- and diacyl-CPG.*** The fatty acid content of SFA, MUFA and PUFA in alkenylacyl-, alkylacyl-, and diacyl-CPG is shown (**Figure 3**). Feeding animals the GG diet increased total PUFA content in alkylacyl-GPC, which rose by 63% compared to animals fed the control diet. No changes occurred in MUFA and SFA for this lipid subclass. Animals fed the GG diet had lower content of PUFA and MUFA and increased content of SFA in diacyl-CPG than observed for control animals. For animals given the SM diet, higher level of PUFA in alkylacyl-CPG lipids was found. These animals also exhibited lower MUFA and higher SFA contents in diacyl-CPG compared to controls.

[00287] ***Changes in total SFA, MUFA and PUFA content of alkenylacyl-, alkylacyl- and diacyl-EPG.*** Animals fed the GG diet exhibited increased content of PUFA in the three EPG lipid subclasses compared to control animals, (increase of 41% in alkenylacyl, 41% in alkylacyl and 30% in diacyl-EPG; **Figure 4**). The increase in PUFA content was accompanied with a decrease in SFA content in alkenylacyl and alkylacyl-EPG. No change was observed in MUFA in alkenylacyl, alkylacyl or diacyl phospholipids. Feeding of the SM diet also resulted in increased content of PUFA and decreased content of SFA in alkenylacyl-EPG and alkylacyl-EPG compared to controls. No effect of the SM diet was detected in diacyl-EPG.

[00288] ***Changes in relative amounts of SFA, MUFA, and PUFA in alkenylacyl, alkylacyl and diacyl phospholipids comprising CPG and EPG classes.*** As PUFA levels in alkylacyl-CPG increased in animals fed the GG diet, there was a concomitant decrease in the ratio of SFA to PUFA in alkylacyl-CPG (2.6 versus 6.8, P<0.05; **Figure 1**). In

contrast, in diacyl-CPG fractions, animals fed the GG diet exhibited an increase in the ratios of SFA to MUFA (5.3 versus 3.6, $P<0.0007$) and SFA to PUFA (1.5 versus 1.1, $P<0.05$).

[00289] Changes in relative amounts of SFA, MUFA, and PUFA in the three subclasses of EPG are illustrated (**Figure 2**). In the GG diet group, alkenylacyl-EPG and alkylacyl-EPG exhibited significantly lower ratios of SFA to PUFA compared to the controls (0.6 versus 1.3 for alkenylacyl-GPE, $P<0.005$, and 0.5 versus 1.2 for alkylacyl-GPE, $P<0.05$). There was also a decrease in the SFA/MUFA ratio (1.8 versus 2.5) in the alkylacyl group. Animals fed the SM diet exhibited a lower SFA/PUFA ratio in the both EPL subclasses compared to animals fed the control diet, but effects were more subtle than that of the GG diet. There was no effect of SM or GG treatments observed on the diacyl-EPG class.

[00290] ***Discussion***

[00291] Previous studies (Merrill *et al.*, 1997; Schmelz *et al.*, 1994; Ogura *et al.*, 1988) showing a possible mechanism to convert gangliosides to EPL in vivo do not explain whether dietary gangliosides can be absorbed and then used for EPL synthesis. The present study confirms that dietary gangliosides can be utilized for biosynthesis of EPL in developing rat intestinal mucosa. There are two possible mechanisms which may explain this effect. Firstly, it is assumed that hexadecanal, a derivative of gangliosides, is directly utilized for the synthesis of EPL as a precursor of ether-linked fatty alcohols [Merrill *et al.*, 1997; Schmelz *et al.*, 1994]. This hypothesis is supported by a study showing that intraperitoneal injection of [^{3}H]GM1, labeled at the C-3 position of sphingosine, is converted into alkylacyl-EPG in the mouse liver [Ogura *et al.*, 1988]. Secondly, a possible indirect mechanism is that reduction of cholesterol in intestinal cells caused by dietary gangliosides may increase EPL synthesis or uptake. Earlier studies show that a decrease in cholesterol content increase EPL uptake in human leukemia cell lines [Diomede *et al.*, 1990; Diomede *et al.*, 1991]. Since experiments in our laboratory have shown a significant decrease in total cholesterol content in the intestine of animals fed the GG diet, it is logical to assume that dietary gangliosides may increase EPL synthesis by decreasing intestinal cholesterol content.

[00292] Several studies have shown that EPL is enriched in neuronal tissues and may play a functional role in neuronal development [Horrocks *et al.*, 1972; Hollan *et al.*, 1998;

Sindelar *et al.*, 1999; and Farooqui *et al.*, 2000]. For example, EPL, especially plasmalogen, dramatically increased during 3-90 days in rat cerebellum development [Leray *et al.*, 1990] and within one year of birth in the human brain [Rouser *et al.*, 1968]. Plasmalogen levels are also significantly higher during cell differentiation in N1E-115 neuroblastoma cells [Murphy *et al.*, 1993]. EPL is a major phospholipid present during synaptogenesis and myelination [Leray *et al.*,1990; Rouser *et al.*, 1968] in which EPL may act as an endogenous antioxidant for membrane peroxidation [Sindelar *et al.*, 1999; Farooqui *et al.*, 2000; and Reiss *et al.*, 1997]. The present results suggest that dietary gangliosides may enhance development of the enteric nerve system during neonatal gut development by providing a means, for increasing EPL content.

[00293] Another possible implication of elevated EPL level is an effect on anti-inflammatory response. Alkylacylglycerol, an analogue of diacylglycerol (DG) which is derived from EPL by phospholipase C (PLC), is known to have a potent inhibitory effect on lipoxygenase [Bauldry *et al.*, 1988] and cytosolic phospholipase A2 (cPLA2) activity [Nixon *et al.*, 1997], both of which stimulate an inflammatory response. Alkylacylglycerol also decreases leukotriene B4 and 5-hydroxyeicosatetraenoic acid (5-HETE) production [Bauldry *et al.*, 1991] by inhibiting PKC activity [Parker *et al.*, 1987] compared to DG. Gangliosides also inhibit PLA2 activity [Basavarajappa *et al.*, 1997]. This hypothesis is supported by our recent study demonstrating that dietary gangliosides decrease platelet activating factor (PAF) and DG in intestinal microdomains. Alkylacyl-phospholipids are mostly localized at the inner membrane [Record *et al.*, 1984], Our results showing a 52% and 49% increase in the level of alkylacyl-CPG and –EPG, respectively, suggest that dietary gangliosides may enhance the inner localization of EPL. This localization of the alkylacyl subclass of EPL at the inner membrane may influence anti-inflammatory response [Bauldry *et al.*, 1988; Nixon *et al.*, 1997; Bauldry *et al.*, 1991; and Parker *et al.*, 1987] by down-regulating cytosolic enzymes and proteins, such as cPLA2 and PKC, known to be inflammatory mediators.

[00294] Higher levels of PUFA in EPL in animals fed the GG diet may have resulted from a decrease in total cholesterol content in intestinal mucosa which is known to cause a corresponding increase of Δ-5 and Δ-6 desaturase activities [Leikin *et al.*, 1988]. Finding that dietary gangliosides promoted a higher level of 20:4n-6, 22:4n-6 and 22:6n-3 in EPG

raises the question of whether these PUFA may serve a particular function in the enteric nervous system as in other neuronal tissues. For example, 22:6n-3 protects retinal photoreceptors by delaying the onset of apoptosis and activates photoreceptor differentiation, promoting opsin expression and inducing apical differentiation in these neurons [Polit *et al.*, 2001]. Supplementation of LC-PUFAs resulted in a resistance against NMDA-induced excitotoxic degeneration of cholinergic neurons in infant rats [Hogyes *et al.*, 2003].

[00295] The present study demonstrates that dietary gangliosides increase total and relative percentage of EPL and the PUFA content of EPL in intestinal mucosa during neonatal development. These results suggest that dietary gangliosides influence gut development and protection by enhancing EPL content which may have a preventative role in carcinogenesis, inflammation, and lipid oxidation. Further investigation is needed to determine if dietary ganglioside also affects the synthesis of EPL in neuronal tissues such as brain, retina and the myenteric plexus in the intestine.

[00296] Example 4

[00297] ***Diet-Induced a Decrease in the Ratio of Cholesterol to Sphingolipids Attenuates the Caveolin and Inflammatory Mediator Content in Microdomains of the Rat Intestine***

[00298] Membrane microdomains rich in cholesterol and sphingolipids including gangliosides are known as cellular binding sites for various pathogens. Cholesterol depletion inhibits the cellular entry of pathogens and also reduces inflammatory signals by disrupting microdomain structure. Our previous study showed that dietary gangliosides increased gangliosides while decreasing cholesterol in the intestinal mucosa. We hypothesized that diet-induced reduction in the cholesterol content in intestinal microdomains disrupts microdomain structure resulting in reduced pro-inflammatory signals. To test this hypothesis, Sprague-Dawley rats (18-day old) were fed semi-purified diets for 2 wks. The control diet contained 20% triglyceride. Experimental diets were formulated by adding either 0.1% ganglioside enriched lipid (GG diet, w/w of diet) or 1.5%, w/w total fatty acids, polyunsaturated fatty acid (PUFA diet, 1% 20:4n-6 and 0.5% 22:6n-3) as triglyceride to the control diet. The ganglioside enriched lipid contained 70-

80% GD3 among the total ganglioside fraction. Levels of cholesterol, ganglioside, caveolin expression and pro-inflammatory mediators, platelet activating factor (PAF) and diglyceride (DG) was measured in microdomains. Feeding animals the GG diet increased GG and decreased cholesterol content in intestinal microdomains by 50% and 23%, respectively. These changes resulted in a significant decrease in the ratio of cholesterol to GG. Increased GD3 and decreased GM3 was found in the intestinal microdomains of animals fed the GG diet in comparison to animals fed the control diet. Caveolin content was significantly reduced in animals fed the GG di*et al*ong with reduction in PAF and DG content in the microdomain. Animals fed the PUFA di*et al*so showed decreased cholesterol, caveolin, PAF and DG content in intestinal microdomains compared to animals fed the control diet, without change occurring in the sphingolipid profile. It is concluded that dietary GG decrease the cholesterol/GG ratio, caveolin, PAF and DG content in microdomains and may have a potential anti-inflammatory effect during gut development.

[00299] The objective of this example was to determine if cholesterol reduction in intestinal mucosa by dietary gangliosides consequently induces decreases in the ratio of cholesterol/sphingolipids, structural disruption of microdomains and ultimately attenuates the level of pro-inflammatory mediators in developing gut.

[00300] MATERIALS AND METHODS

[00301] ***Animals and Diets.*** The experiments presented in this example were approved by the University of Alberta Animal Ethics Committee. Male Sprague- Dawley rats (18-day-old, n=24), average body weight 41.6 ± 1.6 g, were randomly separated into 3 groups of 8 with 2 or 3 rats housed in each polypropylene cage. Animals were maintained at a constant temperature of 23°C and a 12 h light/dark cycle. Animals had free access to water and one of three semi-purified diets containing 20% (w/w) fat for 2 weeks. The composition of the basal diets fed has been previously reported (**Table 5**) (Clandinin *et al.*, 1980). Animal body weight and food intake were recorded every other day throughout the experiment. The control diet (CONT diet) fat was a blend of triglyceride, which reflected the fat composition of an existing infant formula. Fatty acids of the control diet (Jumpsen *et al.*, 1997) were composed of about 31% saturated fatty acids, 48% monosaturated fatty acids and 21% polyunsaturated fatty acids providing a ratio of 18:2n-6 to 18:3n-3 of 7.1. Two

experimental diets were formulated by adding either polyunsaturated fatty acids such as 1% arachidonic acid (20:4n-6) and 0.5% docosahexaenoic acid (22:6n-3) (PUFA diet, 1.5% w/w, Martek Biosciences, USA) or a ganglioside-enriched lipid (GG diet, 0.1% w/w, New Zealand Dairy, New Zealand) to the control diet. Ganglioside enriched lipid consisted of about 45-50% (w/w) phospholipids and 15-20% (w/w) gangliosides. The cholesterol content was negligible (<0.35% w/w total lipid). The ganglioside fraction contained about 80% w/w GD3 and GD1b, GM3 and other gangliosides (GM2, GM1 and GT1b) was 9, 5 and 6% w/w, respectively.

TABLE 5

Composition of Experimental Diets[1]

Diet Treatment	Control	PUFA	GG
Basal diet (g/100g)	80.0	80.0	80.0
Triglyceride	20.0	20.0	19.6
20:4n-6	-	1.0	-
22:6n-3	-	0.5	-
Ganglioside	-	-	0.1
Phospholipid	-	-	0.2
Cholesterol	-	-	tr[2]

[1] The composition of the basal diet has been previously published (Clandinin *et al.*, 1980). The fatty acid composition of the control fat blend is similar to that of an infant formula fat mixture (Jumpsen *et al.*, 1997).
[2] tr presents trace amount.

[00302] ***Collection of Samples.*** After anesthetizing animals with halothane, the small intestine (jejunum to ileum) was excised. The intestine was washed with ice cold 0.9% saline solution to remove visible mucus and dietary debris, opened and moisture was carefully removed with a paper towel to measure mucosa weight. Intestinal mucosa was scraped off with a glass slide on an ice cold glass plate. All mucosa samples were kept in a –70°C freezer until extraction.

[00303] ***Sucrose gradient separation of microdomains.*** Intestinal microdomains were prepared by ultra-centrifugation of a discontinuous sucrose gradient (Igarashi *et al.*, 2000). Intestinal mucosa was suspended with TME (10mM Tris-HCl, 1mM $MgCl_2$, 1mM EGTA)

solution containing 1mM phenylmethyl sulfonyl fluoride, 0.001% w/v apotinin and 2% v/v Triton X-100 for 30min in ice and homogenized with 15 strokes of a Dounce homogeniser with a tight-fitting pestle (Wheaton Scientific, USA). The homogenate was adjusted to 45% w/v sucrose by adding the equal vol of 90% w/v sucrose and then homogenized again with 5 strokes of the Dounce homogeniser. A 5-35% discontinuous sucrose gradient was overlaid on the homogenate in 45% w/v sucrose, which left a 45-35-5% sucrose gradient from the bottom. After 16h centrifugation at 70,000 x g at 4°C in a Beckman SW 28 Ti rotor, interface fraction between 5 and 35% sucrose was collected as the microdomain fraction. Microdomains were washed with TME solution and centrifuged twice at 100,000 x g for 1h at 4°C to remove sucrose and Triton X-100. The pellet was resuspended in phosphate buffer solution and used for protein and lipid analysis. Enrichment of microdomains was demonstrated by testing the amounts of cholesterol and gangliosides in the intestinal microdomain compared to the protein pellet which was soluble in detergent solution. In the intestinal microdomains, cholesterol content was 10 fold higher compared to the detergent soluble proteins. Intestinal gangliosides were exclusively found in the microdomain compared to negative intensity in the detergent soluble protein pellet by a densitometry assay on TLC plates.

[00304] ***Western Blotting for Caveolin Content.*** Protein content from microdomains was measured by QuantiPro BCA™ Assay Kit (Sigma-Aldrich Co. MO. USA). Approximately 25 mg proteins were dissolved with SDS reducing sample buffer and loaded onto 15% SDS-PAGE minigels. After transferring proteins onto nitrocellulose membrane (Amersham Pharmarcia Biotech, UK), membranes were blocked with 5% non-fat dried milk in TBS-T (20 mM Tris; pH 7.6; 137mM NaCl; 0.1% Tween-20) for 1h at room temperature. The primary antibody (BD Biosceince, ON. CA), which specifically recognizes caveolin was diluted in TBS-T with 1% non-fat dried milk (1:1000) and incubated for 90 min at room temperature. The membrane was washed three times for 10 min each time in TBS-T. The secondary antibody (goat anti-mouse IgG-HRP conjugate; Bio-Rad, CA. USA) was diluted in 1% non-fat dried milk in TBS-T (1:2000) and incubated for 1h at room temperature. After washing the membrane with TBS-T three times for 10 min each time, the caveolin protein was developed by enhanced chemiluminescence (ECL) detection reagent according to the protocol supplied by

Amersham Pharmarcia Biotech, UK. Blot intensity of caveolin was measured from five animals from each diet group by using an Imaging Densitometer (Bio-Rad, CA. USA).

[00305] ***Ganglioside Extraction and Purification.*** Total lipid of the microdomain fraction was extracted using the Folch method (Folch *et al.*, 1957). For extraction of gangliosides (Svennerholm, 1964), the upper phase was collected into a test tube and the lower organic phase was washed twice with the Folch upper phase solution (chloroform/methanol/water, 3/48/47 by vol.) to increase GG content isolated from the microdomain fraction. The upper phase gangliosides were pooled and purified by passage through Sep-Pak™ C-18 cartridges (Waters Corporation, Milford, MA, USA) prewashed with 10 ml of methanol, 20 ml of chloroform/methanol (2/1, v/v), and 10 ml of methanol as described by Williams and McCluer (Williams *et al.*, 1980). The upper phase extract was loaded onto Sep-Pak C-18 cartridges. Cartridges were then washed with 20 ml of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5 ml of methanol and 20 ml of chloroform/methanol (2/1, v/v), dried under N_2 gas and then redissolved with 500 ul of chloroform/methanol (2/1, v/v). Gangliosides were stored at -70°C until analysis.

[00306] ***Analysis of Total and Individual Ganglioside Content.*** Total NANA of gangliosides was measured as described by Suzuki (Suzuki, 1964). An aliquot of the ganglioside sample purified using Sep-Pak C-18 cartridges was dried under N_2 gas and dissolved with each of 0.5 ml of distilled H_2O and resorcinol-HCl (Svennerholm, 1957) in screw-capped Teflon-lined tubes. The purple blue color developed by heating was extracted into butylacetate/butanol (85/15, v/v) solvent. Optical density was read by a spectrophotometer (Hewlett Packard, 8452A) at 580 nm. For quantification, N-acetyl neuraminic acid (NANA; Sigma, MO, USA) was used as a standard and total ganglioside content is presented as NANA.

[00307] Individual gangliosides were separated by Silica gel high performance thin layer chromatography (HPTLC; Whatman Inc, Clifton, NJ, USA) using ganglioside standards, GM3, GM2, GD3 and bovine brain ganglioside mixture (Alexis, San Diego, CA, USA) in a solvent system of chloroform/methanol/0.2 % (w/v) $CaCl_2 \cdot 2H_2O$ (55/45/10, by vol.). Individual gangliosides were recovered and measured as described above.

[00308] ***Cholesterol Assay.*** Cholesterol analysis was completed with a test kit (Sigma, MO, USA).

[00309] ***Analysis of Sphingomyelin (SM) and Platelet Activating Factor (PAF).*** Total lipid extracted from microdomains was applied onto a silica gel 'H' TLC plate using chloroform/methanol/2-propanol/0.2% KOH/triethylamine, 45:13.5:37.5:9:27, by vol) and a silica gel 'G' TLC plate (Fisher Scientific, CA) using chloroform/methanol/water, (65:35:6, by vol) for SM and PAF, respectively. Commercial standards of SM, PAF, and lyso-PC (Sigma, MO, USA) were also spotted on the plate for identification. After development, TLC plates were dried, sprayed with 0.1 % ANSA (anilino naphthalene sulfonic acid) and exposed under UV light to detect SM and PAF. Lipids identified were recovered and lipid phosphate was measured (Itoh *et al.*, 1986).

[00310] ***Analysis of Diglyceride (DG).*** To measure DG content, extracted lipid was applied onto a silica gel 'G' TLC plate using a solvent system (petroleum ether/diethyl ether/acetic acid, 80:20:1, by vol). After TLC development, 1,2-DG and 1,3-DG were exposed to 0.1% ANSA and identified under UV light with commercial standards. Cholesterol was recovered with 1,3-DG together as 1,3-DG comigrates with cholesterol. 1,2-DG and 1,3-DG were methylated with a known amount of heptadecanoic acid (C17:0) as an internal standard to measure the total fatty acid amount. To remove cholesterol from 1,3-DG after methylation, fatty acid methyl esters (FAME) were applied onto a silica gel 'G' plate and developed with Toluene. Purified FAME was collected, extracted with hexane and injected into the gas liquid chromatograph (GLC, Varian Model 3400 CX, CA) to measure total fatty acid content in DG. The GLC was equipped with a flame ionization detector and a 25m BP-20 fused capillary column (SGE, Australia).

[00311] ***Statistical Analysis.*** Values shown are means ± standard deviation (SD). Significant difference between the control group and experimental groups was determined by one-way analysis of variance (ANOVA) with SAS. Significant effects of diet treatment were determined by a Duncan multiple range test at a significance level of $p<0.05$.

[00312] RESULTS

[00313] ***Animal Growth and Tissues.*** The initial and final body weight of animals after 2 weeks feeding of experimental diets was not significantly different among Control,

PUFA and GG groups. Intestinal mucosal weight and intestinal length was not affected by dietary treatment. Food consumption was not influenced by diet (data not shown).

[00314] ***Ganglioside Content and Composition.*** The effect of dietary ganglioside on total ganglioside content of intestinal microdomains from animals fed either the CONT or experimental diets for 2 weeks is shown (**Figure 5**-A). Animals fed the GG diet had higher ganglioside content in intestinal microdomains (up to a 50% increase; $P<0.006$) when compared to animals fed the CONT diet.

[00315] **Figure 5** illustrates the total content of GG (A), SM (B) and cholesterol (C) in intestinal microdomains after feeding different diets for 2 wks. Intestinal microdomains were prepared by a discontinuous sucrose density (5-35-45%) ultracentrifugation from mucosa homogenates suspended by TME solution containing 2% v/v Triton-X 100. GG extracted from the Folch upper phase was purified by Sep-Pak C-18 cartridges and used for color densitometry analysis of total NANA content at 580 nm (Suzuki 1964). SM from the lower phase was identified on 'H' TLC plates in chloroform/methanol/2-propanol/0.2% KOH/triethylamine solvent system (45:13.5:37.5:9:27, by vol). Phosphate content in SM was measured by a known method (Itoh *et al.*, 1986). Cholesterol content was analyzed by using a test kit. NANA ($P<0.006$), phosphate ($P<0.004$) or cholesterol ($P<0.02$) content in the microdomain was presented as mg/mg protein. Data were presented as Mean ± SD with n=8 animals in each group.

[00316] Animals fed dietary ganglioside significantly decreased GM3 composition in microdomains compared to animals fed the control diet (83.7% to 77.7%, w/w; **Table 6**) while GD3 increased from 4.4% to 8.3% (w/w; $P<0.0008$). These compositional changes in GM3 and GD3 were accompanied by a significant change in GD3 content ($P<0.002$; **Figure 6-A**) but not in GM3 content. GM1, GD1a, GD1b and GT1b content was not changed by either diet fed (**Table 6**). Animals fed the PUFA diet did not exhibit change in either GG content or composition in microdomains from developing animals. The present result confirms that dietary ganglioside significantly increased total GG content resulting in compositional changes such as decreased GM3 and increased GD3 in neonatal intestinal mucosa.

TABLE 6
Composition of Gangliosides in Rat Intestinal Microdomains Fed Either Control or Experimental Diets[1]

Diet Treatment	Control	PUFA	GG
Ganglioside [2]		(%)[3]	
GM3	83.7 ± 2.7[a]	80.3 ± 4.0[ab]	77.7 ± 3.3[b]
GM1	2.7 ± 0.7	2.1 ± 1.9	2.7 ± 1.4
GD3	4.4 ± 1.0[b]	4.5 ± 1.4[b]	8.3 ± 2.1[a4]
GD1a	3.5 ± 1.0	3.6 ± 1.0	3.5 ± 1.2
GD1b	3.5 ± 1.1	5.6 ± 2.1	4.2 ± 1.9
GT1b	2.2 ± 1.9	3.9 ± 2.1	3.5 ± 0.5

[1] Values are means ± SD of 8 rats. Within a row, values with different superscript letters are significantly different at $P < 0.02$.
[2] Nomenclature was referred from Svennerholm (Svennerholm 1964)
[3] Expressed as a % of total NANA in ganglioside fraction.
[4] Values are significantly different at $P < 0.0008$.

[00317] **Figure 6** shows total content of GD3 (A) and PAF (B) in intestinal microdomains after feeding different diets for 2 wks. Intestinal microdomains were prepared by a discontinuous sucrose density (5-35-45%) ultracentrifugation from mucosa homogenates suspended by TME solution containing 2% v/v Triton-X 100. GG extracted from the Folch upper phase was purified by Sep-Pak™ C-18 cartridges. Individual gangliosides were separated by HPTLC in a solvent system of chloroform/methanol/0.2 % (w/v) $CaCl_2 \cdot 2H_2O$ (55/45/10, by vol.). GD3 was recovered and measured as described (Suzuki 1964). Total lipid extracted from microdomains was applied onto a silica gel 'G' TLC plate using chloroform/methanol/water, (65:35:6, by vol) for PAF. Lipid identified was recovered and phosphate in PAF was measured (43). The amount of GD3 ($P<0.01$) and PAF ($P<0.05$) in the microdomain was presented as mg/mg protein. Data were presented as Mean ± SD with n=8 animals in each group.

[00318] ***Sphingomyelin Content.*** Results show that animals fed the GG diet markedly increased SM content in microdomains by 57% ($P<0.004$), compared to animals fed the CONT diet (**Figure 5**-B). No change in SM content in the microdomain was observed in animals fed either the PUFA diet or the CONT diet. This result demonstrates that dietary ganglioside directly increases SM content in intestinal microdomains in developing animals.

[00319] ***Cholesterol Content of Microdomains.*** Animals fed the GG diet showed significantly lower levels of cholesterol in microdomains, compared to animals fed the CONT diet for 2 wks (**Figure 5-C**). Animals fed the PUFA diet exhibited a lower level of cholesterol in the microdomain, but the effect on cholesterol reduction in the microdomain was smaller than that observed after feeding the GG diet. This result suggests that cholesterol reduction induced by dietary ganglioside in intestinal mucosa is probably due to a decrease in cholesterol content in the microdomain in where cholesterol is enriched.

[00320] ***Ratios of Cholesterol to Gangliosides and Cholesterol to Sphingomyelin.*** Animals fed the GG diet showed a highly significant reduction in the ratio of cholesterol to GG, from 31.3 to 18.9, in intestinal microdomains when compared to animals fed the CONT diet (**Figure** 7-A). Animals fed the PUFA diet did not exhibit a reduced ratio of cholesterol to ganglioside in intestinal microdomains compared to control animals. Animals fed the GG di*et al*so decreased the ratio of cholesterol to SM in intestinal microdomains. The ratio found was 143, 117 and 63 for animals fed the CONT, PUFA, and GG diet, respectively ($P<0.007$; **Figure** 7-B). These observations suggest that dietary ganglioside increased SM content more than GG content in microdomains since the ratio of cholesterol to SM was more dramatically reduced by 56% in microdomains while the ratio of cholesterol to GG was decreased by 40% when compared to control animals.

[00321] **Figure 7** shows the ratio of cholesterol/GG (A) and cholesterol/SM (B) in intestinal microdomains after feeding different diets for 2 wks. Intestinal microdomains were prepared by a discontinuous sucrose density (5-35-45%) ultracentrifugation from mucosa homogenates suspended by TME solution containing 2% v/v Triton-X 100. GG extracted from the Folch upper phase was purified by Sep-Pak C-18 cartridges and used for color densitometry analysis of total NANA content at 580 nm (Suzuki 1964). SM from the lower phase was identified on 'H' TLC plates in chloroform/methanol/2-

propanol/0.2% KOH/triethylamine solvent system (45:13.5:37.5:9:27, by vol). Phosphate content in SM was measured by a known method (Itoh *et al.*, 1986). Cholesterol content was analyzed by using a test kit. The ratio was obtained by dividing cholesterol content by either GG ($P<0.05$) or SM ($P<0.01$) content (mg/mg protein) in the microdomain. Data were presented as Mean ± SD with n=8 animals in each group.

[00322] ***Caveolin Content in Microdomains.*** Animals fed the GG diet or the PUFA diet exhibited significantly lower expression of caveolin protein in intestinal microdomains compared to animals fed the CONT diet (**Figure 8-A**). The blot intensity of caveolin in animals fed the GG and the PUFA diet was 55% and 45% lower, respectively, than that observed for animals fed the CONT diet (**Figure 8-B**).

[00323] **Figure 8** shows caveolin content determined by western blotting (A), and the intensity of blots (B) in intestinal microdomains fed control diet or treatment diets for 2 wks. Intestinal microdomains were prepared by a discontinuous sucrose density (5-35-45%) ultracentrifugation from mucosa homogenates suspended by TME solution containing 2% v/v Triton-X 100. Proteins lysed (approximately 25mg/lane) in SDS reducing sample buffer loaded onto 15% SDS-PAGE minigels and immunoblotted with anti-caveolin-1 antibody. A: lane 1, caveolin standard (21-24 kDa); lane 2-5, Control diet; lane 6-9; PUFA diet; lane 10-13, GG diet. B: Blot intensity was analysed by an Imaging Densitometer and each data represents the Mean ± SD derived from five animals (n=5) fed different diets.

[00324] ***Content of Inflammatory Mediators, Diglyceride (DG) and Platelet Activating Factor(PAF).*** DG and PAF content in microdomains was measured to determine if dietary ganglioside has potential anti-inflammatory effects resulting from reduction of pro-inflammatory signals. Animals fed either the GG diet or PUFA diet showed lower levels of 1,2-DG and total DG content, but not in 1,3-DG, in microdomains compared to control animals (**Table 7**). Animals fed the GG diet significantly decreased 1,2-DG and total DG by 44% and 43% of controls, respectively. Animals fed the PUFA diet exhibited a smaller reduction compared to control animals (33% and 32% for 1,2-DG and total DG, respectively). Animals fed either the GG diet or the PUFA diet showed significantly lower levels of PAF in microdomains when compared to the animals fed the CONT diet (**Figure 6-B**). Feeding the GG or the PUFA diet decreased PAF content up to 59% and 47%,

respectively. Taken together, these results suggest that dietary ganglioside and PUFA have potential anti-inflammatory effects in developing animals and that dietary ganglioside is more effective in reducing two inflammatory factors than feeding PUFA.

TABLE 7

Content of 1,2-, 1,3-, and Total Diglyceride in Rat Intestinal Microdomains Fed Either Control or Experimental Diets[1]

Diet Treatment	Control	PUFA	GG
1,2-diglyceride[2]	15.4 ± 2.3[a]	10.4 ± 3.3[b]	8.6 ± 4.4[b]
1,3-diglyceride	1.7 ± 0.2	1.3 ± 0.6	1.2 ± 0.2
Total diglyceride	17.1 ± 2.2[a]	11.7 ± 3.8[b]	9.8 ± 4.6[b]

[1] Values are means ± SD of 8 rats. Within a row, values with different superscript letters are significantly different at $P < 0.009$.
[2] Content was expressed as ug/mg protein.

[00325] DISCUSSION

[00326] Diet Induced-Change. Diet induced-change in the microdomain GD3 and GM3 Content is significant because GD3 and GM3 are involved in cellular function. GM3 is co-localized with signalling molecules such as c-Src, Rho, and Fak in microdomains (Iwabuchi *et al.*, 2000; Yamamura *et al.*, 1997). GD3 activates T-cells (Ortaldo *et al*, 1996) and has an anticarcinogenic effect in the mouse colon (Schmelz *et al.*, 2000) Thus, these results may suggest that decreased GM3 alters signals related to these molecules and that increased GD3 (**Figure 6**-(A)) enhances immune function and gut protection during development. Our results showing accumulation of dietary gangliosides in the microdomains are supported by a previous study demonstrating that administration of [^{3}H]GM3 to Neuro 2a cells showed enrichment of [^{3}H]GM3 in microdomains (Prinetti *et al.*, 1999). These observations suggest that exogenous supplementation of gangliosides directly incorporates into the microdomain.

[00327] Cholesterol is an important lipid involved in compartmentalizing microdomains with lipids and proteins (Incardona *et al.*, 2000). Recent studies found that viral pathogens

and cholera toxin appear to reach the ER by caveolae (Lencer *et al.*, 1995; Majoul *et al.*, 1996). However, cholesterol reduction in cell membranes inhibits the invasion of HIV-1 (Popik *et al.*, 2002), cholera toxin (Wolf *et al.*, 2002), and malarial parasite (Samuel *et al.*, 2001) by disruption of microdomain structure. Cholesterol depletion by drugs down-regulates caveolin gene expression (Hailstones *et al.*, 1998). The present study confirmed that diet-induced cholesterol reduction in the microdomain also decreased caveolin protein expression. The present data therefore suggests a potential anti-infective effect of dietary ganglioside by reducing cholesterol content leading to less caveolin expression.

[00328] The present study provides new information indicating dietary ganglioside decreased pro-inflammatory DG and PAF signals in intestinal microdomains in developing animals. DG located in microdomains modulates the structure and function of microdomains through PKC (Liu *et al.*, 1995; Smart *et al.*, 1995). PAF binds to a PAF receptor in cell membranes to initiate inflammatory signalling events (Flickinger *et al.*, 1999). Together, these observations suggest that PAF may colocalize in microdomains as either ether phospholipids or with its receptors and that diet-induced increase in gangliosides or decrease in cholesterol or caveolin in the microdomain may disrupt PAF and DG localization in the microdomain, thereby resulting in inhibition of inflammatory signalling events.

[00329] This example shows that a physiological level of dietary ganglioside has anti-inflammatory effects in developing animals. Thus present results also illustrate that gangliosides have a protective role in gut development in infants.

[00330] Example 5

[00331] ***Dietary Gangliosides and Long-Chain PUFA Alter GD3 and Phospholipids in Neonatal Rat Retina***

[00332] Dietary long-chain polyunsaturated fatty acids (LCP) such as arachidonic acid (AA) and docosahexaenoic acid (DHA) have been shown to improve visual acuity in infants (Birch *et al.*,1998; Carlson *et al.*, 1996; Faldella *et al.*, 1996; and Hoffman *et al.*,2003). It is thought that dietary LCP stimulate neonatal retinal development by altering membrane phospholipids, which in turn affect cell signaling pathways (Giusto *et al.*, 1997; Huster *et al.*, 2000). During early development, the ganglioside composition of the retina

also changes significantly whereby GD3 becomes the primary ganglioside in mammalian retina (Daniotti *et al.*, 1994; Daniotti *et al.*, 1990). Since gangliosides play an important role in neuronal cell differentiation and proliferation (Byrne *et al.*, 1983; Fujito *et al.*, 1985; and Ledeen*et al.*, 1998), this change in ganglioside profile may indicate retinal maturation. Here we show that a ganglioside diet enriched in GD3 increases ganglioside content by 39% in neonatal rat retina, with a relative increase in GD3. Furthermore, we demonstrate that dietary AA and DHA significantly increase the relative levels of GD3 in the retina of neonatal rats, providing evidence that dietary LCP affects ganglioside metabolism in the developing retina and suggesting a new mechanism by which these dietary lipids may promote maturation of photoreceptor cells.

[00333] INTRODUCTION

[00334] An unusual simplified ganglioside composition is observed in adult retinal photoreceptor cells, compared to that in other central nervous system-derived neurons (Dreyfus *et al.*, 1996). Changes in specific ganglioside content occurs within photoreceptor cells during postnatal maturation to reach an end stage characterized by a predominance of GD3 in the outer retina and only trace amounts of less complex gangliosides (Dreyfus *et al.*, 1996). Since GD3 is the most prevalent ganglioside in fully mature mammalian retinas (Daniotti *et al.*, 1990; Dreyfus *et al.*, 1996), it can be used as a biological marker to evaluate the stage of retinal development. Although the majority of gangliosides are localized in the inner retinal membranes, GD3 is primarily found in photoreceptors in the outer retina (Dreyfus *et al.*, 1996; Dreyfus *et al.*,1997), where it plays an important role increasing membrane permeability and fluidity (Barbour *et al.*, 1992; Seyfried *et al.*, 1985).

[00335] In embryonic chicken retina, total gangliosides increase up to three-fold from the 8-day-old embryo to the 15-day-old stage while GD3 content decreases by 50% Daniotti *et al.*, 1994). In rats, GM1 is downregulated and GD1b is upregulated during the period corresponding to formation of the outer segment, a time which correlates with onset of retinal function (Fontaine *et al.*, 1998). The outer segments, photoreceptor cells, synaptic cells and rhodopsin kinase in the rat retina become functionally active between 10 and 30 days after birth (Fontaine *et al.*, 1998; Ho *et al.*, 1986) during which time GD3 becomes

the predominant ganglioside (Daniotti *et al.*, 1990; Dreyfus *et al.*, 1996; Dreyfus *et al.*, 1997).

[00336] Dietary long-chain polyunsaturated fatty acids (LCP) such as docosahexaenoic acid (DHA) and arachidonic acid (AA) influence the lipid composition of retinal membranes, particularly during developmental stages (Birch *et al.*, 1998; Carlson *et al.*, 1996; Faldella *et al.*, 1996; Anderson *et al.*, 1976; Carrie *et al.*, 2002; and Suh *et al.*, 1994).

[00337] Dietary DHA alters the lipid composition of neuronal tissues in retina and brain, affecting the turnover time for rhodopsin in photoreceptor membranes (Carrie *et al.*, 2002; and Suh *et al.*, 1994). Dietary DHA increases DHA levels, and levels of other very long-chain fatty acids in rod outer segment membranes in young rats, whereas it does not change the fatty acid composition of these retinal membranes in mature rats (Nishizawa *et al.*, 2003; Xi *et al.*, 2003). Inclusion of DHA and AA in infant formulas improves visual development and acuity in infants, but little is known about the biological basis for this effect. Gangliosides are associated with neuronal cell differentiation and proliferation processes including migration, neurite outgrowth, axon generation, and synapse formation (Byrne *et al.*,1983; Fujito 1985; Ledeen *et al.*, 1998; and Mendez-Otero *et al.*, 2003), processes crucial to visual maturation, but the influence of dietary lipids on retinal ganglioside composition during development is poorly understood.

[00338] In this example, we hypothesized that dietary gangliosides and LCP could exert biological effects on visual development through modification of retinal gangliosides, which change in composition during maturation. To test this hypothesis, we devised a study that would explore whether an LCP-enriched diet (LCP diet) or a ganglioside-enriched diet (GG diet) could alter retinal ganglioside composition during early development of neonatal rats. We found that the GG diet caused a 39% increase in the total retinal ganglioside content, indicating that gangliosides can be absorbed and incorporated into body tissues (**Figure 9**).

[00339] More unusual was the finding that the LCP diet as well as the GG diet caused significant increases in the relative proportion of GD3, by 19% and 13%, respectively (**Table 8**). The specific increase in this particular ganglioside may indicate that dietary ganglioside or LCP stimulate neuronal development in neonates through enhancing the

expression of GD3, with possible implications for neonatal development of visual neural pathways and photoreceptor cell function.

[00340] Concurrent with changes in ganglioside profile was an alteration in retinal phospholipid composition attributed to both the GG and the LCP diets. Both diets were associated with increases in the relative amounts of phosphatidylinositol and lyso-phosphatidylethanolamine, and a decrease in phosphatidylserine and phosphatidylcholine (**Table 9**). There was no effect of the GG diet on total phospholipids, whereas the LCP diet was associated with a decrease in total retinal phospholipids (**Figure 9**). All other parameters measured, including total retinal cholesterol content, remained unchanged with diet treatment. Phospholipid turnover alters electric surface potential by affecting calcium and cation concentration in retinal rod outer segments (Huster *et al.*, 2000) and is tightly regulated by light and phosphorylation-dephosphorylation reactions (Giusto *et al.*, 1997). Thus, compositional changes in retinal phospholipids in response to dietary ganglioside or LCP may affect light adaptation and activation of protein kinases, which ultimately may lead to enhanced development of retinal function in neonates.

[00341] Our study demonstrates that even small physiologic amounts of dietary gangliosides or LCP can have profound effects on the lipid profile of membranes within the developing retina. Further study is needed to assess the mechanism by which these dietary lipids exert the observed effects on retinal lipid composition and to determine whether other neural tissues are similarly affected.

[00342] MATERIALS AND METHODS

[00343] ***Animals and Diets.*** Two experimental diets or a control diet were fed to male weanling (18-day-old, 40 + 4.5 g) Sprague-Dawley rats for two weeks. Animals had free access to water and one of three semi-purified diets containing 20% (w/w) fat. The control diet was formulated as 80% basal diet 25 plus 20% fat as a triglyceride blend, reflecting the overall fat composition of infant formula. The LCP-enriched diet (LCP diet) was formulated by including 1% AA and 0.5% DHA by weight as triglyceride in the control diet. The ganglioside-enriched diet (GG diet) was formulated by including in the control diet 0.1% by weight of a ganglioside-enriched powder (70-80% GD3, 9% GD1b, 5% GM3, 6% other gangliosides). The GG diet also contained 0.2% (w/w) phospholipid and

0.07% (w/w) cholesterol. Body weight and food intake were measured every other day throughout the experimental period.

[00344] ***Collection of Retina, Lipid Extraction and Ganglioside Separation.*** After decapitation of animals, whole retinas were removed. All samples were weighed and kept in a -70°C freezer until analysis. Total lipids were extracted using the Folch method (Folch *et al.*, 1957). Gangliosides were extracted into the Folch upper phase solution. The lower organic phase was washed twice with chloroform/methanol/water (3/48/47, v/v/v) and the upper phase extracts combined. Gangliosides were purified by passing the upper phase extract through Sep-Pak™ C18 cartridges (Waters Corporation, Milford, MA) preconditioned with 10 mL of methanol, 20 mL of chloroform/methanol (2/1, v/v), and 10 mL of methanol. Cartridges were then washed with 20 mL of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5 mL of methanol and 20 mL of chloroform/methanol (2/1, v/v), dried under N_2 gas and then redissolved with 500 mL of chloroform/methanol (2/1, v/v). Gangliosides were stored at -70°C until analysis.

[00345] ***Analysis of Ganglioside Content.*** Measurement of total gangliosides as N-acetyl neuraminic acid (NANA) was performed as described by Suzuki (1964). An aliquot of the purified ganglioside sample was dried under N_2 gas and dissolved with 0.5 mL each of distilled H_2O and resorcinol-HCl (Svennerholm, 1957). The purple-blue color developed by heating was extracted into butyl acetate/butanol (85/15, v/v). Optical density was read at 580 nm. Commercial NANA (Sigma, MO) was used as a standard.

[00346] Individual gangliosides were separated by silica gel high performance thin layer chromatography (HPTLC; Whatman Inc, Clifton, NJ) in a solvent system of chloroform/methanol/0.2 % (w/v) $CaCl_2 \cdot 2H_2O$ (55/45/10, v/v/v) and identified using ganglioside standards GM3, GM2, GD3 and bovine brain ganglioside mixture (Alexis, San Diego, CA). Individual gangliosides were recovered and measured as described above.

[00347] ***Analysis of Phospholipid Content.*** Phospholipids were separated from total retinal lipids by thin layer chromatography (TLC) on silica gel 'G' (Fisher Scientific, CA) using chloroform/methanol/water, (65:35:6, by vol.). Individual phospholipids were resolved on silica gel 'H' TLC plates using chloroform/methanol/2-propanol/0.2%

KOH/triethylamine (45:13.5:37.5:9:27, by vol). and identified by comparison to authentic phospholipid standards (Sigma, MO). Plates were visualized by 0.1 % anilinonaphthalene sulfonic acid under UV exposure. Lipid fractions were recovered and lipid phosphate was measured according to the method of Itoh *et al.*, (1986).

[00348] ***Statistical analysis.*** Six retinas from three animals were pooled to constitute one replicate to analyze retinal lipids because of the small amount of lipids in the retina. Values presented are mean ± standard deviation (SD) from 6 replicates (n = 6) except for individual phospholipid analysis (n = 5). Significant differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with SAS (SAS Institute Inc, V6, Fourth Edition, Cary, NC). Significant effects of diet treatment were determined by a Duncan multiple range test at a significant level of $P < 0.05$.

[00349] RESULTS

[00350] ***Animal Growth and Tissues.*** The initial and final body weight of animals, food consumption or retina weight after 2 weeks feeding of experimental diets was not different among groups.

[00351] ***Ganglioside Content, Composition and Ratio of GT1b to GD3 in Retina.*** Animals fed the GG diet increased total ganglioside content in the retina by 39% ($P<0.001$) when compared to animals fed the CONT diet, indicating bioavailability of dietary ganglioside. Feeding animals either the LCP or the GG diet showed an increase in the relative percentage of GD3 in the retina in comparison with feeding animals the CONT diet (by 19% and 13%, respectively). The composition of GM3, GM1, GD1a, GD1b and GT1b was not changed by either diet fed. The ratio of GT1b to GD3 in the retina was reduced in the both LCP and GG fed animals compared to animals fed the CONT diet.

[00352] **Figure 9** illustrates the total content of a) gangliosides and b) phospholipids in the retina of control and treatment groups. Values are the mean ± SD of 6 samples. Asterisks represent significant difference at levels of $p < 0.0008$ and $p < 0.001$, respectively.

Table 8

Percent composition of gangliosides in the retina of animals fed different diets[1]

Ganglioside [2]	Control	LCP	GG	Significant effect

									of diet (p < x)
GM3	7.6	±	2.8	5.8	±	3.2	7.7	± 2.3	
GM1	7.3	±	1.8	6.8	±	2.0	7.7	± 2.2	
GD3	25.0	±	1.8[b]	29.8	±	1.7[a]	28.2	± 3.5[a]	0.01
GD1a	14.3	±	3.3	15.4	±	3.5	16.1	± 1.8	
GD1b	19.1	±	2.2	19.0	±	2.4	16.7	± 1.0	
GT1b[3]	26.9	±	2.9	23.2	±	3.1	23.6	± 1.3	

[1] Values are mean ± SD where n = 6 for each group. Percentage of gangliosides was measured as % of total NANA content (μg/retina) in the ganglioside fraction.

[2] Nomenclature of gangliosides is described by Svennerholm.

[3] GT1b fraction included GQ1b fraction because of close proximity during TLC separation.

[00353] ***Phospholipid Composition.*** Feeding animals the LCP diet, but not the GG diet, reduced total phospholipid content in the retina compared to animals fed the CONT diet. Animals fed either the LCP diet or the GG diet showed lower levels of phosphatidylinositol and lyso-phosphatidylethanolamine (PE) and higher levels of phosphatidylserine and phosphatidylcholine (PC) compared to animals fed the CONT diet. PE and sphingomyelin (SM) were not changed by either diet treatment fed. Ratios of PE to PC, major phospholipids in the retina, and PC to SM were not affected by both the LCP and GG diet treatment.

Table 9

Percent composition of phospholipids in the retina of animals fed different diets[1]

Phospholipid[2]	Control			LCP			GG			Significant effect of diet (p < x)
PE	35.2	±	4.7	33.4	±	1.5	34.4	±	2.3	
PI	7.4	±	1.6[a]	5.1	±	1.3[b]	4.6	±	0.7[b]	0.01
PS	2.3	±	0.4[b]	2.8	±	0.4[a]	3.1	±	0.1[a]	0.01
LPE	8.3	±	0.6[a]	6.6	±	0.4[b]	6.4	±	1.0[b]	0.001
PC	40.4	±	3.3[b]	45.5	±	2.8[a]	45.6	±	2.7[a]	0.04
SM	6.5	±	1.3	6.6	±	2.2	6.0	±	0.7	

[1] Values are the mean ± SD for n = 5 in each group. Percentage of phospholipids was measured as phosphate content (μg/retina) in the phospholipid fraction.
[2] PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, LPE = lysophosphatidylethanolamine, PC = phosphatidylcholine, SM = sphingomyelin

[00354] ***Cholesterol Content.*** Cholesterol content in animals fed either the LCP or GG diet was not different from that of animals fed the CONT diet. Animals fed dietary gangliosides exhibited a highly significant reduction in the ratio of cholesterol to gangliosides and phospholipid to gangliosides compared to retinas of animals fed the CONT diet, (28%, $P<0.005$ and 30%, $P<0.0003$, respectively). Feeding animals the LCP diet reduced the ratio of cholesterol to gangliosides and phospholipids to gangliosides, but not the ratio of cholesterol to SM, compared to animals fed the CONT diet. The ratio of cholesterol to phospholipids in animals fed the LCP increased compared to animals fed the CONT diet. No change was observed in the ratio of cholesterol to phospholipid and cholesterol to SM in animals fed the GG diet compared to controls.

[00355] DISCUSSION

[00356] Dietary GG or LCP modifies the lipid classes and the composition of gangliosides and phospholipids in the developing retina. For example, animals fed the LCP diet increased the relative percentage of GD3, but not total ganglioside content, compared to animals fed the CONT diet. The increase in the relative percentage of GD3 was accompanied with significant changes in total and individual phospholipids. The effect of dietary LCP on the compositional change of GD3 may suggest that dietary LCP influence activity of GD3 synthase, an enzyme in the outer retina required to synthesize GD3 from GM3 (Daniotti *et al.*, 1992). Trafficking of DHA-containing PL from the trans-Golgi network to the retina outer segment is accompanied with rhodopsin (Rodriguez *et al.*, 1997). Sphingolipids including GG are enriched in microdomains called lipid rafts or caveolae, which are important domains for lipid trafficking. Thus, the present study suggests that diet-induced increase in GD3 induced by the LCP diet may influence the trafficking of DHA and rhodopsin from the trans-Golgi network to the outer segment of the retina. This result also imply that beneficial effects known that dietary LCP influence visual acuity by altering the LCP composition in the retinal membrane may be a synergistic effect of the compositional change of gangliosides in the retina.

[00357] In the retina of the rat, the outer segments, photoreceptor cells, synaptic cells and rhodopsin kinase become functionally active between 10 days and 1 month after birth (Fontaine *et al.*, 1998; Ho *et al.*, 1986) while GD3 becomes the predominant ganglioside (Daniotti *et al.*, 1990; Dreyfus *et al.*, 1997; and Dreyfus *et al.*,1996). GD3 in the outer

retina is involved in increasing membrane permeability and fluidity and is enriched in differentiated retinas. Since animals used in the present study were fed for 2 wks from 17 days of age, this study suggests that dietary LCP and GG may stimulate retinal maturation and development by increasing GD3 content.

[00358] Change in the ratio of cholesterol to gangliosides may induce signal transduction for retinal development as known functional involvement of lipid microdomains. The finding that animals fed the LCP or the GG diet showed significant changes in the ratio of cholesterol to gangliosides suggests structural and functional changes of microdomains in retinal membranes. For instance, administration of gangliosides into the plasma membrane of MDCK cells displaces GPI-anchored signaling proteins from microdomains (Simons *et al.*, 1999). Exogenous addition of [^{3}H]GM3 to mouse neuroblastoma Neuro2a cells shows enrichment of [^{3}H]GM3 in microdomains resulting in induction of neuritogenesis by c-SRC activation (Prinetti *et al.*, 1999).

[00359] In summary, this study demonstrates that dietary LCP and gangliosides modify metabolism of phospholipids and gangliosides in developing retinal membranes. The present study indicates that a small physiologic amount of phospholipids or gangliosides has a profound effects on the lipid profile of membranes in the retina. The bioavailability of gangliosides in the diet is high rapidly altering the GD3 composition in structural components of the photoreceptor membrane. Dietary gangliosides would thus alter ganglioside content in other neuronal cell types.

[00360] Example 6

[00361] ***Diet Induces Change in Membrane Gangliosides in the Intestinal Mucosa, Plasma and Brain***

[00362] In this example the role of gangliosides in plasma membranes of mammalian cells as biologically important molecules is examined. Gangliosides are involved in cell differentiation, proliferation, neuritogenesis, growth, inhibition, signaling and apoptosis (Byrne *et al.*, 1983; De Maria *et al.*, 1997; Ledeen ,1989; and *et al.*,1996). GM32 and GM1 can act as receptors for enterotoxins such as rotavirus in animals (Rolsma *et al.*, 1998) and Vibrio cholerae and Escherichia coli in humans (Laegreid *et al.*, 1987). GD3 stimulates T-cell activation in human peripheral blood lymphocytes (Ortaldo *et al.*, 1996;

Welte *et al.*,1987). Ganglioside content and composition is significantly different between stages of development. For example, in human brain, GM1 increases from birth to the age of 1 year while GD1a decreases in the white matter (Vanier *et al.*, 1971). The ratio of GM3/GD3 is about 0.2-0.3 in human colostrum while the ratio is greater than 3 in mature milk since GM3 gradually becomes a major ganglioside upon lactation (Takamizawa *et al.*, 1986). Recent studies have also shown neuroprotective effects of GM1. GM1 treatment of neonatal rats prevents hypoxic damage (Krajnc *et al.*, 1994). GM1 intervitreally injected is protective against rat retinal ischemia induced by pressure (Mohand-Said *et al.*, 1997), and intravenous administration of GM1 reduces infarct volume caused by focal cerebral ischemia (Lazzaro *et al.*, 1994). Despite evidence that gangliosides are involved in development, it is still not clear if dietary gangliosides induce changes in membrane gangliosides or where GM3 and GD3 are localized in the enterocyte membrane. This information is vital to understanding the biological functions of these molecules during the period of development in which their role is the most significant.

[00363] The cholesterol content of membrane is also important in maintaining an optimal cell membrane environment. Recent work shows that cholesterol homeostasis is related to sphingomyelin content (Slotte 1999), and cholesterol absorption is regulated in part by sphingomyelin content in intestinal cell membranes (Chen *et al.*, 1992). Cholesterol is enriched in membrane microdomains such as rafts and caveolae, perhaps mediating signal transduction (Maekawa *et al..*, 1999). Gangliosides have the same ceramide as the anchored hydrophobic moiety of sphingomyelin, with the only difference occurring in a polar head group. No studies have reported the effect of gangliosides on cholesterol turnover or membrane content of cholesterol in vivo.

[00364] The present research was designed to determine whether dietary ganglioside increases the content of total and individual gangliosides and affects the level of cholesterol, thereby changing the ratio of cholesterol to gangliosides in the intestinal mucosa, plasma and brain in developing rats. This is also the first study showing the localization of GM3 and GD3 in the enterocyte membrane.

[00365] MATERIALS AND METHODS

[00366] ***Animals and diets.*** Male 18-day-old Sprague Dawley rats (n = 24), average body weight 40 ± 4.5 g, were randomly separated into 3 groups of 8 with 2 or 3 rats housed in

each polypropylene cage. Animals were maintained at a constant temperature of 23°C and a 12 h light/dark cycle. Animals had free access to water and one of three semi-purified diets containing 20% (w/w) fat for 2 weeks. The composition of the basal diets fed has been reported (Clandinin *et al.*, 1980). Animal body weight and food intake were recorded every other day throughout the experiment. The control diet fat was a blend of triglyceride, which reflected the fat composition of an existing infant formula. Dietary fatty acids were composed of about 31% saturated fatty acids, 48% monounsaturated fatty acids and 21% polyunsaturated fatty acids with a ratio of 18:2n-6 to 18:3n-3 of 7.1. Two experimental diets were formulated by adding either sphingomyelin (SM, 1% w/w, Sigma, MO, USA) or a ganglioside-enriched lipid (GG, 0.1% w/w, New Zealand Dairy, New Zealand) to the control diet. Ganglioside-enriched lipid consisted of about 45-50% (w/w) phospholipids and 15-20% (w/w) gangliosides. The cholesterol content was <0.35% w/w total lipid. The ganglioside fraction contained about 80% w/w GD3, with GD1b, GM3 and other gangliosides accounting for 9, 5 and 6% w/w, respectively.

[00367] ***Collection of Samples.*** After anesthetizing animals with halothane, blood was collected by cardiac puncture and immediately spun at 1000 x g (JA-20 Rotor, Beckman, USA) for 30 min to recover plasma. Following decapitation, the brain and small intestine (jejunum to ileum) were excised. The intestine was washed with ice-cold 0.9% saline solution to remove visible mucus and dietary debris, opened and moisture was carefully removed with a paper towel to correctly measure mucosa weight. Intestinal mucosa was scraped off with a glass slide on an ice-cold glass plate. All mucosa samples were weighed and kept in a –70°C freezer until extraction.

[00368] ***Immunofluorescence study.*** Intestinal sections were collected from animals. Samples were washed with cold phosphate buffered saline (4°C), cut into 5 mm pieces and fixed with 4% paraformaldehyde in PBS for 1 h at 4°C. After washing with cold PBS, samples were infiltrated with 15% and 30% sucrose in PBS for 90 min and overnight, respectively, at 4°C for cryostat protection. The tissue sections were placed on plastic molds and covered with embedding medium by optimal cutting temperature (O.C.T.; Tissue Tek, Sakura Finetek USA) on dry ice. Frozen sections (1 mm thickness) were mounted on polylysine-coated microscope slides and washed in cold PBS for 30 min at room temperature. The sections were blocked with 2% bovine serum albumin in PBS for 1

h at room temperature and then incubated with anti-ganglioside GM3 (Mouse IgM, Seikagaku Co., USA) (diluted 1:25), or anti-ganglioside GD3 monoclonal antibody (Mouse IgM, Seikagaku Co., USA) (diluted 1:25), for 2 h at room temperature. After washing the sections 3 times for 10 min with cold PBS, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (Sigma, MO, USA) (diluted 1:300) for 1 h at room temperature in the dark room and washed with PBS again 3 times for 10 min. After staining, a drop of N-propyl gallate was added onto the section before mounting a cover slip. All samples were sealed with nail polish and examined with a confocal microscope (Zeiss Confocal Laser Microscope 510, Carl Zeiss, Germany) with an Argon laser line (488nm excitation, barrier filter LP505, Plan-Neofluar 40X, 1.3 oil immersion objective).

[00369] ***Ganglioside extraction and purification.*** Total lipid was extracted using the Folch method (Folch *et al.*,1957). For extracting gangliosides, the lower phase was washed twice with Folch upper phase solution (chloroform/methanol/water, 3/48/47 by vol.). The upper phase gangliosides were pooled and then purified by passing through Sep-Pak™ C18 cartridges (Waters Corporation, Milford, MA, USA) prewashed with 10 mL of methanol, 20 mL of chloroform/methanol (2/1, v/v), and 10 mL of methanol as described by Williams and McCluer (1980). The upper phase extract was loaded onto C18 cartridges. Cartridges were then washed with 20 mL of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5 mL of methanol and 20 mL of chloroform/methanol (2/1, v/v), dried under N_2 gas and then redissolved with 500 mL of chloroform/methanol (2/1, v/v). Gangliosides were stored at -70°C until analysis.

[00370] Analysis of total and individual ganglioside content by measuring NANA

[00371] Total NANA-gangliosides were measured as described by Suzuki (1964). An aliquot of the ganglioside sample purified by Sep-Pak C18 cartridges was dried under N_2 gas and dissolved with each of 0.5 mL of distilled H2O and resorcinol-HCl (Svennerholm 1957) in screw-capped Teflon-lined tubes. The purple blue color developed by heating was extracted into butylacetate/butanol (85/15, v/v) solvent. Optical density was read by a spectrophotometer (Hewlett Packard, 8452A) at 580 nm. For quantitative analysis, N-acetyl neuraminic acid (Sigma, MO, USA) was used as a standard.

[00372] Individual gangliosides were separated by silica gel high performance thin layer chromatography (HPTLC; Whatman Inc, Clifton, NJ, USA) along with standards of ganglioside GM3, GM2, GD3 and bovine brain ganglioside mixture (Alexis, San Diego, CA, USA) in a solvent system of chloroform/methanol/0.2 % (w/v) $CaCl_2 \cdot 2H_2O$ (55/45/10, by vol.). Individual ganglioside fractions were scraped off and measured as described above.

[00373] ***Cholesterol assay.*** Cholesterol analysis was completed with a test kit (Sigma, MO, USA).

[00374] ***Statistical analysis.*** The values shown are means ± standard deviation (SD). Significant differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with SAS. Significant effects of diet treatment were determined by a Duncan multiple range test at a significance level of $p<0.05$.

[00375] RESULTS

[00376] ***Animal growth and tissues.*** There were no significant differences among the control, SM and GG groups either in terms of the initial body weight of animals or their final weight after 2 weeks feeding of experimental diets (**Table 10**). Brain weight, intestinal mucosal weight and intestinal length were not affected by dietary treatment. Food consumption was not influenced by diet.

Table 10

Weight of Animals and Tissues Fed Control or Experimental Diets[1]

Diet Treatment:	Control	SM	GG
Initial Body Wt. (g)	39.9 ± 4.5	40.6 ± 4.4	40.3 ± 4.5
Final Body Wt. (g)	117 ± 13.5	118 ± 12.1	120 ± 13.1
Intestine Length (cm)	82.0 ± 5.8	79.8 ± 6.2	84 ± 6.0
Mucosa Wt. (g)	2.1 ± 0.3	2.2 ± 0.3	2.1 ± 0.3
Brain Wt.(g)	1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.1

[1]Values are mean ± SD with 8, 8, and 6 for mucosa, plasma and brain, respectively.

[00377] ***The localization of GM3 and GD3 in the enterocyte by confocal microscopy.*** Localization of GM3 and GD3 in the enterocyte was determined using a confocal microscope. GM3 stained with FITC-conjugate was almost exclusively localized at the apical membrane of the enterocyte (**Figure 10**). The majority of the GD3 was found in the basolateral membrane of the enterocyte with only minor staining in the apical membrane (**Figure 10**).

[00378] **Figure 10** shows immunofluorescent detection of GM3 localization. Immunofluorescent detection of GM3 in intestinal villi was analyzed with (B, D) or without (A, C) treatment of anti-GM3 visualized with FITC-conjugate IgM and confocal microscopy. GM3 was almost exclusively localized at the apical membrane of enterocytes.

[00379] **Figure 11** shows Immunofluorescent detection of GD3 localization. Immunofluorescent detection of GD3 in intestinal villi was analyzed with (B, D) or without (A, C) treatment of anti-GD3 visualized with FITC-conjugate IgM and confocal microscopy. GD3 was mostly localized at the basolateral membrane with minor staining at the apical membrane of enterocytes.

[00380] ***Total ganglioside content in tissues and plasma.*** The effect of dietary ganglioside on total ganglioside content of the intestinal mucosa, plasma and brain from animals fed the control and experimental diets for 2 weeks are shown (**Figure 12**). Animals fed the GG diet had significantly higher ganglioside content in the intestinal mucosa, plasma and brain compared to control animals. The highest tissue level of ganglioside was observed in the intestinal mucosal membrane. The lowest level of ganglioside was found in brain membrane. No change in total ganglioside content of either tissues or plasma was found after feeding the SM diet.

[00381] **Figure 12** illustrates the effect of dietary treatment on total content of gangliosides in (A) the intestinal mucosa, (B) plasma and (C) brain for animals fed either the control or experimental diet for two weeks. Values are means ± SD, $p<0.02$ for A and C, and $p<0.003$ for B. Treatment values represent the means of $n = 7$, 8 and 6 animals for mucosa, plasma and brain, respectively.

[00382] ***Individual ganglioside composition in tissues and plasma.*** Animals fed the GG diet showed a higher level of GD3 and GQ1b in the intestinal mucosal membrane compared to control animals (**Table 11**, $p<0.001$ and $p<0.05$, respectively). This result

was accompanied by significant reduction of ganglioside GM3, which is normally a major ganglioside in the intestinal mucosa. Feeding the GG diet did not affect the level of GM2, GM1, GD1a, GD1b or GT1b in the intestinal mucosa compared to the control. Animals fed the SM diet did not exhibit any change in individual ganglioside patterns, but showed increase in GM2 compared to control animals ($p<0.05$).

Table 11

Composition of Gangliosides in the Rat Intestinal Mucosa Fed Either Control or Experimental Diets[1]

Diet Treatment:	Control	SM	GG
Ganglioside[2]		(%)[3]	
GM3	83.5 ± 6.7[a]	82.4 ± 7.5[a]	76.4 ± 6.9[b]
GM2	2.0 ± 0.9[b]	4.1 ± 2.4[a]	2.8 ± 0.8[ab]
GM1	2.7 ± 1.7	3.0 ± 1.7	1.7 ± 1.2
GD3[4]	3.2 ± 1.3[b]	2.2 ± 0.9[b]	7.5 ± 1.9[a]
GD1a	2.3 ± 1.5	2.8 ± 1.5	1.9 ± 0.9
GD1b	1.9 ± 1.0	1.5 ± 0.8	2.2 ± 1.4
GT1b	2.1 ± 2.2	1.6 ± 1.6	3.1 ± 2.2
GQ1b	2.3 ± 1.6[b]	2.6 ± 1.6[b]	4.5 ± 1.6[a]

[1] Values are mean ± SD of 7 rats. Within a row, values with different superscript letters are significantly different at $P < 0.05$.

[2] Nomenclature was referred from Svennerholm.

[3] Expressed as a % of total ganglioside fraction. [4]Values aresignificantly different at $P < 0.001$.

[00383] In plasma, only four major ganglioside fractions (GM3, GD1a, GD1b and GT1b) were measured since the total ganglioside content was much lower compared to either tissue. The GD3 fraction could not be quantified because unknown fraction partially overlapped with GD3. Two minor gangliosides, GM2 and GM1, were faintly visible on the TLC plate. Animals fed the GG or SM diet did not show a significant change in the individual ganglioside composition of plasma compared to control animals (**Table 12**). There was a trend toward increased GM3 in animals fed the GG diet compared to control animals ($p<0.07$). GD1a and GD1b represented 33.9% to 36.0% and 16.1 to 19.6% of the plasma ganglioside fraction, respectively.

Table 12

Composition of Gangliosides in Plasma of Rats Fed Either Control or Experimental Diets[1]

Diet Treatment:	Control	SM	GG
Ganglioside		(%)[2]	
GM3	21.6 ± 3.3	26.5 ± 3.5	28.5 ± 5.3
GD1a	36.0 ± 5.5	30.2 ± 3.6	33.9 ± 2.7
GD1b	16.3 ± 1.8	19.6 ± 6.2	16.1 ± 3.5
GT1b	26.1 ± 2.2	23.7 ± 4.1	21.4 ± 5.5

[1] Values are mean ± SD of 5 samples.

[2]Expressed as a % of the total ganglioside fraction.

[00384] Brain ganglioside fractions were separated into 14 fractions as shown by Sonnino *et al.* (1983). The five major gangliosides were GD1a, GT1b, GD1b, GQ1b and GM1. Minor components were GT1a, GD3 and GM3. The remaining six fractions were collected as others. Animals fed the GG diet or SM diet exhibited no change in individual ganglioside composition in the brain compared to control animals (**Table 13**), but the total ganglioside content increased.

Table 13

Composition of Gangliosides in Brain of Rats Fed Either Control or Experimental Diets[1]

	DIET TREATMENT	CONTROL	SM
GANGLIOSIDE		(%)[2]	
GM3	2.5 ± 0.8	2.8 ± 1.5	2.6 ± 0.5
GM1	6.3 ± 0.3	6.0 ± 0.3	6.2 ± 0.5
GD3	3.0 ± 0.7	3.0 ± 0.3	3.0 ± 0.5
GD1a	23.2 ± 2.8	24.0 ± 2.0	23.6 ± 2.5
GT1a	4.3 ± 0.4	4.5 ± 0.3	4.8 ± 0.5
GD1b	11.3 ± 0.8	11.0 ± 1.2	11.4 ± 0.9
GT1b	22.4 ± 2.5	21.7 ± 1.6	21.9 ± 0.9
GQ1b	6.8 ± 0.3	6.5 ± 0.1	6.4 ± 0.6
Others[3]	20.3 ± 4.4	20.5 ± 3.8	20.0 ± 3.4

[1] Values are mean ± SD of 4 rats.

[2] Expressed as a % of the total ganglioside fraction.

[3] Six minor fractions were gathered as others.

[00385] ***Cholesterol content in tissues and plasma.*** Animals fed the GG diet showed a lower level of cholesterol in the intestinal mucosa and brain, but not in the plasma, compared to animals fed the control diet (**Figure 13**). Animals fed the SM diet did not exhibit any change in total cholesterol content in the plasma and brain, but exhibited a significant difference in the intestinal mucosa compared to control animals ($p<0.03$). Unlike a previous report (Slotte 1999), the SM diet did not increase cholesterol content in the intestinal membrane, but reduced cholesterol content compared to animals fed the control diet. Animals fed the GG diet showed lower cholesterol in plasma compared to those fed the SM diet (**Figure 13B**).

[00386] **Figure 13** illustrates the effect of dietary treatment on cholesterol content in (A) the intestinal mucosa, (B) plasma and (C) brain of animals fed either the control or experimental diets for two weeks. Values are means ± SD, $p<0.03$ for A and B, and $p<0.0002$ for C. Treatment values represent the means of $n = 7$, 8 and 6 animals for mucosa, plasma and brain, respectively

[00387] ***Ratio of cholesterol to gangliosides.*** Animals fed the GG diet showed a highly significant reduction in the ratio of cholesterol to ganglioside in the intestinal mucosa, plasma and brain compared to animals fed the control diet (**Figure 14**), and a lower level of cholesterol in plasma compared to feeding SM, which is an appropriate single lipid control. Animals fed the SM di*et al*so exhibited a reduced ratio of cholesterol to ganglioside in the intestinal mucosa and brain, but not in plasma compared to control animals. Of the three dietary treatments, the lowest ratio of cholesterol to ganglioside was observed in animals fed the GG diet. In contrast, the highest ratio was found in animals fed the control diet in both tissues but not in the plasma. In the plasma, the highest ratio of cholesterol to gangliosides was observed in animals fed the SM diet.

[00388] **Figure 14** illustrates the effects of dietary treatment on the ratio of cholesterol to ganglioside in (A) the intestinal mucosa, (B) plasma and (C) brain of animals fed either the control or experimental diets for two weeks. Data values are means ± SD, A: $p<0.0007$, B: $p<0.002$ and C: $p<0.0001$. Treatment values represent the means of $n = 7$, 8

and 6 animals for mucosa, plasma and brain, respectively. The ratio was obtained by dividing the tissue cholesterol content (mg/g wet weight) by the total tissue content of ganglioside (mg/g wet weight).

[00389] DISCUSSION

[00390] The notion that gangliosides may have beneficial effects in development has prompted studies of the influence of dietary ganglioside on intestinal and brain development. GM1 acts as a receptor for cholera toxin and E. coli. GM3 is the major ganglioside in the enterocyte of humans and animals (Holgersson *et al.*, 1988; Bouhours *et al.*,1983) but the intracellular localization is not known. The present study clearly shows that gangliosides GM3 and GD3 are localized at the apical and basolateral membrane of the enterocyte, respectively. As GM3 is the major ganglioside in the enterocyte of humans and animals, the present study suggests that the different localizations of GM3 and GD3 probably have different biologic and/or physiologic functions for protection and development. GM1 bound with cholera toxin is transcytosized from the apical to the basolateral membrane to activate the basolateral effecter, adenylate cyclase (Lencer *et al.*, 1995). The present study did not examine the possibility that GM3, like GM1, may also be transcytosized. Total ganglioside content and individual ganglioside composition were significantly changed, but the degree of change could not be quantitatively estimated by confocal microscopy.

[00391] Dietary ganglioside significantly increases membrane ganglioside content in the intestinal mucosa, plasma and brain, thereby having potential to cause developmental change. Increased membrane ganglioside in the intestinal mucosa might influence enterocyte immune functions since gangliosides activate immune functions and provide attachment sites for enterotoxins and viruses. Neonatal intestinal mucosa has a relatively low level of immunoglobulin-containing cells after birth to about 2 weeks of age (Perkkio *et al.*, 1980). Mother's milk and the intestine have a compensatory high level of gangliosides during this period (Bouhours *et al.*, 1983; Carlson 1985), suggesting that gangliosides may have a key role in protection of the neonate from antigens.

[00392] In the present example, increased membrane ganglioside was accompanied by changes in the individual ganglioside composition of the intestinal mucosa. GD3 was increased by feeding gangliosides while the major ganglioside in the intestine, GM3,

decreased compared to control animals. Since GD3 activates T-cells and has an anticarcinogenic effect in the mouse colon (Schmelz *et al.*, 2000), it is logical to suggest that increased GD3 might influence enterocyte functions and infection by altering the interaction with the developing immune system. In plasma, diet treatment considerably increased total gangliosides, but no change was found in the composition of individual gangliosides. In contrast to human serum, where GM3 is the major ganglioside (Senn *et al.*, 1989), GD1a was the major ganglioside in rat plasma. Dietary ganglioside markedly increased total ganglioside content in the brain compared to animals fed the control diet. The present data suggests that dietary ganglioside may affect brain development since an increase of ganglioside content in the brain may effect protection against neuronal injury, induce neurite growth, and is observed in well-fed animals compared to undernourished animals (Karlsson *et al.*, 1978; Morgan *et al.*, 1980). The present study agrees with previous results describing the pattern of major brain gangliosides. In rodents, GM1 increases from the 3rd to 24th month (Aydin *et al.*, 2000) during development (Sun *et al.*, 1972). The low level of GM1 observed in the present study may be due to the younger animal age compared to that of previous work. The lack of significant change in individual ganglioside patterns observed in the brain may be attributed to a short experimental period (2 weeks), the lack of change in ganglioside patterns in the plasma or specific control of individual ganglioside composition in the brain.

[00393] The SM content used in this experiment was relatively much higher (>10 fold) than the ganglioside content of the GG diet. This higher level of dietary SM did not alter tissue ganglioside content, suggesting that dietary GG is a better source for enhancing membrane GG and that dietary SM may not be used for GG synthesis during the early stage of development.

[00394] The present example indicates that animals fed the GG diet exhibited a significant reduction of cholesterol content in the intestinal mucosa compared to animals fed the control diet. A disruption in microdomain structures caused by reduced cholesterol content may prevent endocytosis of toxins or invasions by bacteria (Parpal *et al.*, 2001; Samuel *et al.*, 2001). A similar result was also observed for animals fed the SM diet. Long term feeding of 1% sphingolipid in the diet significantly reduces plasma cholesterol content (Kobayashi *et al.*, 1997). Our study, in agreement with Imaizumi (Imaizumi *et al.*,

1992), showed that feeding SM for two weeks did not alter plasma cholesterol content. This observation may be due to differences in age, diet, species or *in vivo* and *in vitro* experimental conditions.

[00395] Animals fed the GG diet showed a significant decrease in cholesterol in the brain compared to animals fed the control diet. Cholesterol is maintained in the brain by regulating its de novo synthesis and the uptake of LDL-cholesterol as well as the release of HDL-cholesterol (Bastiaanse *et al.*, 1997). Cholesterol turnover takes place very slowly in brain (Andersson *et al.*, 1990). In comparison with Kobayashi *et al.*, (1997), our data suggest that the effect of gangliosides on cholesterol reduction in the brain may be dependent on NANA combined with glycosphingolipid since sphingolipids, such as cerebroside and SM that do not contain NANA, do not affect brain cholesterol content.

[00396] It appears that individual gangliosides have different roles in the regulation of cell behaviour, as each ganglioside is localized in different enterocyte membrane sites. Cholesterol is an important factor involved in cell permeability, fluidity (Rietveld *et al.*, 1998), gap junctions (Malewicz *et al.*, 1990) and membrane microdomains called rafts or caveolae (Brown *et al.*, 1998). Change in membrane cholesterol content in the intestinal mucosa and brain might affect membrane functions during development.

[00397] The ratio of cholesterol to gangliosides was decreased in the intestinal mucosa, plasma and brain by feeding the GG diet compared to animals fed the control diet. Changes in this ratio, in both tissues and plasma, could suggest that dietary gangliosides alter membrane functions. This suggestion is supported by early studies showing that changes in membrane lipid composition (Clandinin *et al.*, 1991) and membrane cholesterol content (Rietveld *et al.*, 1998) influence membrane functions. The present research also suggests that dietary gangliosides might affect the traffic of lipids and proteins in membrane microdomains since GM3 and GD3 are localized in different sites and (glyco)sphingolipids including gangliosides and cholesterol are the most abundant lipids present in rafts and caveolae (Incardona *et al.*, 2000; Parton, 1994). The functions of caveolae are closely involved with cholesterol content (Incardona *et al.*, 2000). Depletion of cholesterol content in caveolae inhibited the MAP kinase complex, stimulated Erk enzymes and increased mitogenesis (Furuchi *et al.*, 1998).

[00398] Gene expression of caveolin, a marker of protein for caveolae, was upregulated with cholesterol (Fielding *et al.*, 1997), suggesting that cholesterol-protein interaction directly modulates gene expression important for cell development and behaviour. In the sarcolemma, reduction in cholesterol content results in increased Ca^{2+}/Mg^{2+}-ATPase activity and decreased Ca^{2+}/Mg^{2+}-ATPase activity when the level of cholesterol is high (Ortega *et al.*, 1984). It is also logical to suggest that dietary ganglioside might influence intestinal immune functions by modulating the lipid profile in membrane lipid rafts since activation of signal transduction by IgE receptors and T-cell receptors is dependent on membrane lipid rafts (Moran *et al.*, 1998; Stauffer *et al.*, 1997).

[00399] In summary, this study suggests that dietary gangliosides are absorbed by the intestine, remodelled in the enterocyte and induce changes in membrane total content of ganglioside and cholesterol in the intestinal mucosa and brain. The observations suggest that dietary ganglioside fed at a physiological level will alter membrane lipid profiles that influence membrane functions involved in a wide variety of cell functions in neonatal development. Infant formulas have lower levels of gangliosides and a different ganglioside composition compared to that of human breast milk (Pan *et al.*, 2000; Sanchez-Diaz *et al.*, 1997). The bioavailability of dietary gangliosides demonstrated in this paper and the impact on the lipid composition of developing tissues indicates that these differences in feeding regimens are of biological importance.

[00400] Example 7

[00401] ***Dietary Ganglioside: Functions in the Intestine During Development***

[00402] This example illustrates the role of dietary gangliosides in decreasing inflammatory factors PAF and DG in microdomains of the rat intestine. The intestines of rats fed either a control diet (Cont) or a diet high in gangliosides (GG), as described in previous Examples, were assessed for GM3 and GD3 in microdomains. The methodology used for assessing ganglioside composition is as described in previous Examples.

[00403] The increase in GD3 composition of microdomains for animals fed the ganglioside-enriched diet correlated to a decrease in both PAF and DG in microdomains. These parameters are indicative of a decrease in inflammatory factors in the intestine and thus show inflammation mediation induced by dietary gangliosides.

[00404] **Figure 15** illustrates the composition of GM3 and GD3 in microdomains of rat intestine. While GM3 is reduced on the GG diet, GD3 is increased.

[00405] **Figure 16** illustrates the composition of PAF and DG in microdomains of rat intestine. Both PAF and DG are reduced with a GG diet. Data shown is presented on a μg/mg protein basis.

[00406] **Figure 17** illustrates the caveolin content of microdomains for animals fed a control, PUFA or GG diet. The presence of caveolin is reduced for both the PUFA and GG diets, relative to control. This decrease in caveolin protein, a marker of microdomains, was observed in rat intestines from animals fed a ganglioside-enriched diet. A reduction in caveolin is also indicative of a reduced likelihood of bacterial and/or viral infection through an intestinal entry route.

[00407] **Example 8**

[00408] ***Dietary Gangliosides Effect Plasma Lipid Content and Ratios of Plasma Lipids***

[00409] This example illustrates the effect of dietary gangliosides on decreasing plasma lipid content, specifically cholesterol and triglyceride, as well as the lipid ratios cholesterol: NANA and cholesterol: phosphorus in the rat.

[00410] Plasma was sampled from rats fed either a Control, SM or GG (high in ganglioside) diet, as described in previous Examples. Plasma NANA, phosphorus, cholesterol, and triglyceride, was evaluated as described in previous Examples.

[00411] **Figure 18** illustrates that feeding a diet high in ganglioside resulted in decreased plasma cholesterol and triglyceride. This finding suggest reduced cholesterol and lipid absorption from the intestine. Further, because of the decreased cholesterol and increased NANA with the GG diet, a striking reduction in the cholesterol: NANA ratio was observed, illustrating that the GG diet effects lipid composition in plasma.

[00412] **Example 9**

[00413] ***Preparation of a Crude Fraction Enriched in Natural Gangliosides***

[00414] This example provides a formulation of natural gangliosides which may be used according to an embodiment of the invention. The crude fraction described is derived from whole milk, and in particular, fat globules from whole milk.

[00415] The initial step in preparing the crude fraction involved in MFGM isolation involves separating the fat globules from fresh, uncooled milk. Centrifugation at low g forces readily separates all but the smallest fat globules from milk, and helps to minimize physical damage to the fat globules (Patton *et al.*, 1975; Mather, 1987). The second step in the process is to wash the fat globules, this will remove entrained or adsorbed components of milk serum as well as caseins and whey (Keenan *et al.*, 1988). Washing involves resuspension and reflotation of lipid globules in buffered or unbuffered water made isotonic with milk serum by addition of sucrose or sodium chloride (Keenan *et al.*, 1988). Two wash cycles at temperatures above 25°C is sufficient to remove caseins and whey proteins from globules prepared in a laboratory centrifuge, while milk separated via a mechanical cream separator may require 3 or 4 washes (Keenan *et al.*, 1988). The final composition of the material that is recovered as MFGM will vary according to the method and extent of washing of lipid globules (Keenan *et al.*, 1995), and thus intermittent analytical testing carried out to ensure that the product yield and composition conform to specifications.

[00416] The final steps in the procedure release the membrane from the globule via slow and successive freeze/thaw cycles. FInally, the membrane is pelleted via centrifugation at 100 000 x g for 60-90 mins (Patton *et al.*, 1975).

[00417] Small milk batches of 10 L can be used to test the method during the intial phases of progressing to 100 L, and finally to 1000 L batches.

[00418] The above-described embodiments of the present invention are intended to be examples only. Alterations, modifications and variations may be effected to the particular embodiments by those of skill in the art without departing from the scope of the invention, which is defined solely by the claims appended hereto.

Claims:

1. A formulation comprising at least one ganglioside for mediating inflammation.

2. The formulation according to claim 1 wherein mediating inflammation comprises mediating inflammation of the intestine, retina or neuronal tissue.

3. A formulation according to claim 1 wherein mediating inflammation comprises preventing or treating an inflammatory disease.

4. The formulation according to claim 3, wherein said inflammatory disease is selected from the group consisting of inflammatory bowel disorders, disorders arising from allergic responses, and diseases involving epithelial surface responses.

5. The formulation according to claim 1, wherein said ganglioside is selected from the group consisting of: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid.

6. The formulation according to claim 1 in the form of a supplemented liquid or food.

7. The formulation according to claim 6, wherein said supplemented liquid or food comprises infant formula or infant foods.

8. The formulation of claim 1 wherein the at least one ganglioside comprises about 80% GD3, 9% GD1b, and 5% GM3 on a weight/weight basis.

9. The formulation of claim 1 wherein one ganglioside selected from the group consisting of GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid, comprises more than 50% of ganglioside content.

10. A method for mediating inflammation in a subject in need thereof comprising the step of providing at least one ganglioside to said subject for oral consumption.

11. The method according to claim 10 wherein mediating inflammation comprises mediating inflammation of the intestine or retina.

12. The method according to claim 10 wherein mediating inflammation comprises preventing or treating an inflammatory disease.

13. The method according to claim 12, wherein said inflammatory disease is selected from the group consisting of inflammatory bowel disorders, disorders arising from allergic responses, and diseases involving epithelial surface responses.

14. The method according to claim 10, wherein said ganglioside is selected from the group consisting of: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid.

15. Use of at least one ganglioside for preparation of a medicament for oral consumption to mediate inflammation in a subject in need thereof.

16. The use according to claim 15, wherein inflammation comprises inflammation of the intestine, retina or neuronal tissue.

17. The use according to claim 15 wherein to mediate inflammation comprises preventing or treating an inflammatory disease.

18. The use according to claim 15, wherein said ganglioside is selected from the group consisting of: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid.

19. A formulation comprising at least one ganglioside for reducing plasma cholesterol level.

20. The formulation according to claim 19, wherein said ganglioside is selected from the group consisting of: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid.

21. The formulation according to claim 19 in the form of a supplemented liquid or food.

22. The formulation according to claim 21, wherein said supplemented liquid or food comprises infant formula or infant foods.

23. The formulation of claim 19 wherein the at least one ganglioside comprises about 80% GD3, 9% GD1b, and 5% GM3 on a weight/weight basis.

24. The formulation of claim 19 wherein one ganglioside selected from the group consisting of GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid, comprises more than 50% of ganglioside content.

25. A method for reducing blood cholesterol in a subject in need thereof comprising the step of providing at least one ganglioside to said subject for oral consumption.

26. The method according to claim 25, wherein said ganglioside is selected from the group consisting of: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid.

27. Use of at least one ganglioside for preparation of a medicament for oral consumption to reduce blood cholesterol in a subject in need thereof.

28. The use according to claim 27, wherein said ganglioside is selected from the group consisting of: GD3, GM1, GM2, GM3, GD1b, NANA, and sialic acid.

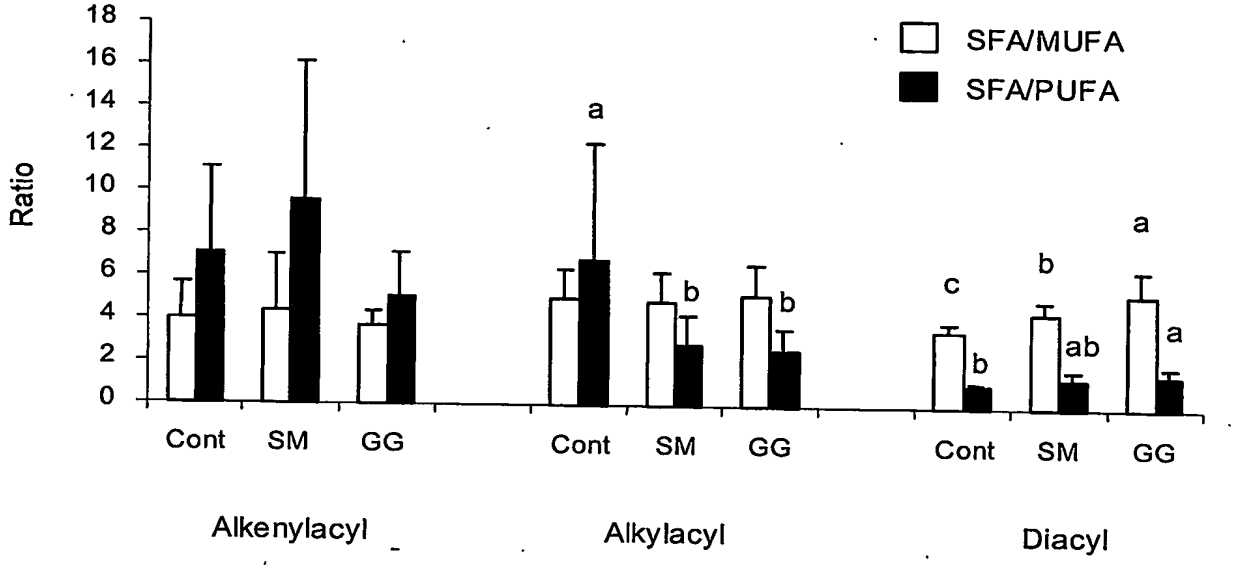
Ratio
18
16
14
12
10
8
6
4
2
0
SFA/MUFA
SFA/PUFA
a
b
b
c
b
a
b
ab
a
a
Cont
SM
GG
Cont
SM
GG
Cont
SM
GG
Alkenylacyl
Alkylacyl
Diacyl

FIG. 1

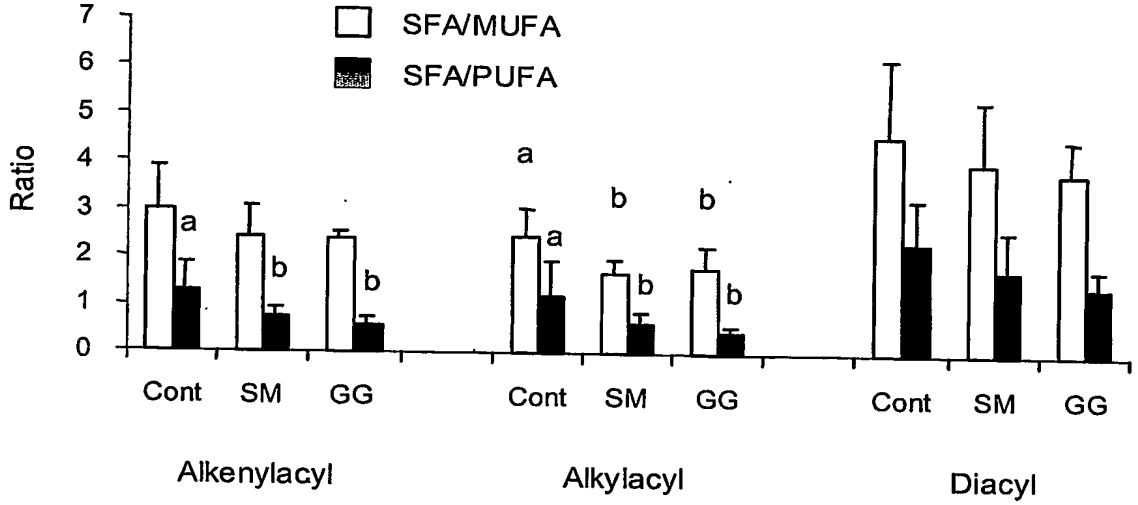
Ratio
7
6
5
4
3
2
1
0
SFA/MUFA
SFA/PUFA
a
b
b
a
b
b
a
b
b
Cont
SM
GG
Cont
SM
GG
Cont
SM
GG
Alkenylacyl
Alkylacyl
Diacyl

FIG. 2

Fatty acid composition of alkenylacyl, alkylacyl and diacyl subclasses in CPG in intestinal mucosa of animals fed control diet or treatment diets[1]

	Alkenylacyl-CPG			Alkylacyl-CPG			Diacyl-CPG		
	Control	SM	GG	Control	SM	GG	Control	SM	GG
C 14:0	6.2 ± 1.6	8.1 ± 5.4	5.0 ± 1.3	5.4 ± 2.1	6.1 ± 2.5	6.4 ± 2.5	0.8 ± 0.2	1.1 ± 0.3	0.9 ± 0.2
C 14:1	1.9 ± 1.5^{a}	0.5 ± 0.3^{b}	0.4 ± 0.6^{b}	1.0 ± 0.7	0.3 ± 0.4	0.5 ± 0.5	-	-	-
C 16:0	33.7 ± 5.8	32.2 ± 8.6	32.3 ± 8.2	34.3 ± 7.3	33.3 ± 6.1	33.0 ± 4.3	21.0 ± 1.7^{b}	24.4 ± 2.2^{a}	24.3 ± 2.5^{ef}
C 16:1(7)	1.4 ± 1.6	0.8 ± 0.5	1.1 ± 1.6	0.4 ± 0.6	0.7 ± 0.6	0.4 ± 0.4	0.4 ± 0.0	0.3 ± 0.2	0.2 ± 0.1
C 18:0	25.5 ± 3.7	23.2 ± 5.9	22.0 ± 6.7	25.8 ± 3.6^{a}	17.6 ± 5.7^{b}	19.0 ± 4.6^{bf}	22.7 ± 1.1^{b}	24.8 ± 3.4^{b}	27.7 ± 3.2^{ef}
C 18:1(9)	8.8 ± 4.3	8.5 ± 4.3	5.9 ± 2.3	8.7 ± 3.1	7.7 ± 1.5	7.4 ± 1.3	11.6 ± 0.8^{a}	10.5 ± 0.8^{b}	9.2 ± 1.1^{cf}
C 18:2(6)	3.2 ± 1.8	3.2 ± 2.1	2.4 ± 1.6	5.3 ± 4.1	5.5 ± 1.8	5.5 ± 2.9	30.0 ± 1.6^{a}	26.6 ± 3.1^{b}	23.5 ± 3.4^{cf}
C 18:3(6)	0.5 ± 0.4	1.0 ± 1.2	0.7 ± 0.8	0.8 ± 0.6	0.7 ± 0.4	0.6 ± 0.7	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
C 18:3(3)	0.5 ± 0.6	0.3 ± 0.2	0.6 ± 0.6	0.5 ± 0.9	0.1 ± 0.2	0.4 ± 0.3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C 20:0	1.2 ± 1.2	1.0 ± 0.4	1.9 ± 1.7	1.0 ± 0.2	0.8 ± 0.4	0.9 ± 0.6	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
C 20:1	0.3 ± 0.4	0.6 ± 0.3	1.0 ± 0.7	0.4 ± 0.3	0.4 ± 0.4	0.6 ± 1.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
C 20:2	0.0 ± 0.1	0.1 ± 0.1	0.7 ± 1.1	0.1 ± 0.3	0.1 ± 0.2	2.6 ± 3.0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
C 20:3(6)	0.3 ± 0.4	0.6 ± 0.7	0.4 ± 0.5	0.5 ± 0.6^{b}	1.3 ± 1.3^{a}	0.0 ± 0.1^{bf}	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
C 20:4(6)	1.8 ± 0.6^{ab}	1.1 ± 0.5^{b}	3.1 ± 2.1^{a}	5.7 ± 3.7	9.5 ± 4.8	9.3 ± 6.7	9.4 ± 0.4	8.8 ± 2.8	10.2 ± 1.5
C 20:3(3)	0.3 ± 0.4	0.9 ± 0.8	0.7 ± 0.8	0.4 ± 0.4	0.4 ± 0.4	0.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C 20:5(3)	1.5 ± 0.9	0.6 ± 0.6	2.2 ± 2.0	0.7 ± 0.6	0.8 ± 0.5	1.0 ± 0.5	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
C 22:0	0.5 ± 0.4^{b}	2.6 ± 1.5^{a}	2.2 ± 2.1^{ab}	1.3 ± 0.8	2.2 ± 1.3	1.8 ± 1.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2
C 22:1(9)	1.3 ± 1.0	1.5 ± 2.3	1.5 ± 1.6	0.1 ± 0.2^{b}	1.2 ± 1.0^{ab}	1.7 ± 1.5^{a}	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C 22:2(6)	2.1 ± 1.1^{ab}	1.5 ± 0.9^{b}	4.0 ± 2.9^{a}	0.0 ± 0.0^{b}	2.9 ± 2.6^{a}	1.2 ± 1.4^{abf}	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.1
C 22:4(6)	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.4	0.7 ± 0.9	0.6 ± 0.8	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.0
C 24:0	2.4 ± 1.5	3.3 ± 1.5	3.2 ± 2.2	1.8 ± 1.0^{a}	1.8 ± 1.0^{a}	0.6 ± 0.8^{b}	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
C 22:6(3)	1.2 ± 1.1	0.9 ± 1.1	tr[2]	1.2 ± 1.0^{b}	2.7 ± 0.9^{a}	4.2 ± 1.8^{af}	1.4 ± 0.2	1.1 ± 0.4	1.3 ± 0.2
C 24:1(9)	5.3 ± 2.9	7.5 ± 5.4	8.8 ± 3.6	4.0 ± 1.8^{a}	3.0 ± 0.7^{ab}	1.8 ± 1.4^{b}	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
SFA[3]	69.5 ± 7.3	70.4 ± 10.9	66.6 ± 6.6	69.6 ± 10.6	61.8 ± 9.3	61.7 ± 7.8	45.2 ± 2.3^{b}	50.7 ± 5.5^{a}	53.6 ± 5.8^{ef}
MUFA	19.1 ± 4.8	19.4 ± 7.3	18.7 ± 2.6	14.6 ± 2.5	13.2 ± 1.9	12.4 ± 2.2	12.8 ± 0.9^{a}	11.5 ± 0.9^{b}	10.2 ± 1.1^{cf}
PUFA	11.4 ± 3.6	10.2 ± 5.2	14.8 ± 4.9	15.8 ± 8.9^{b}	25.0 ± 7.6^{a}	25.9 ± 6.6^{a}	42.0 ± 1.7^{a}	37.7 ± 5.7^{ab}	36.2 ± 4.8^{b}

[1] Means ± SD (% w/w) in 3 subclasses from 7, 8 and 7 animals, for the control, SM and GG group, respectively. Within a row, values with different superscript letters are significantly different at P< 0.05. Superscript letters with [a,b] and [a,f] are significantly different at P<0.01, P<0.001, and P<0.0001, respectively. [2] tr represents trace amount.
[3] SFA, MUFA and PUFA represent saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, respectively.

FIG. 3

Fatty acid composition of alkenylacyl, alkylacyl and diacyl subclasses in EPG in intestinal mucosa of animals fed control diet or treatment diets[1]

	Alkenylacyl-EPG			Alkylacyl-EPG			Diacyl-EPG		
	Control	SM	GG	Control	SM	GG	Control	SM	GG
C 14:0	3.9 ± 1.3	3.1 ± 1.1	3.1 ± 1.2	4.4 ± 1.5	3.0 ± 1.9	2.5 ± 1.5	0.7 ± 0.4	0.4 ± 0.1	0.5 ± 0.2
C 14:1	0.7 ± 0.3	0.9 ± 1.0	0.5 ± 0.4	0.7 ± 0.6^{a}	0.2 ± 0.2^{b}	0.2 ± 0.1^{b}	-	-	-
C 16:0	19.0 ± 3.6^{a}	15.4 ± 1.7^{b}	14.2 ± 2.3^{bf}	18.7 ± 5.4^{a}	12.7 ± 3.2^{b}	11.4 ± 1.9^{bf}	9.8 ± 2.5	8.5 ± 1.8	8.1 ± 1.9
C 16:1(7)	2.1 ± 1.3	2.5 ± 0.7	2.3 ± 0.6	0.6 ± 0.5	0.3 ± 0.2	0.3 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
C 18:0	17.6 ± 4.7^{a}	14.4 ± 4.1^{ab}	11.6 ± 2.7^{b}	14.8 ± 3.2^{a}	11.2 ± 4.0^{b}	9.8 ± 2.1^{b}	46.9 ± 8.6	43.5 ± 7.4	42.6 ± 4.3
C 18:1(9)	9.0 ± 2.0	8.2 ± 1.2	7.3 ± 0.9	12.0 ± 3.1	11.6 ± 1.9	9.2 ± 1.3	12.2 ± 2.9	13.1 ± 2.1	12.8 ± 1.5
C 18:2(6)	4.0 ± 1.3	4.6 ± 0.5	4.1 ± 0.7	5.4 ± 1.0	6.4 ± 0.6	5.6 ± 0.8	11.8 ± 3.3	14.7 ± 3.0	14.2 ± 1.8
C 18:3(6)	0.6 ± 0.3^{a}	0.6 ± 0.1^{a}	0.3 ± 0.1^{b}	0.5 ± 0.4	0.5 ± 0.3	0.4 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
C 18:3(3)	2.2 ± 1.2	2.1 ± 0.9	2.1 ± 1.0	0.5 ± 0.3	0.6 ± 0.3	0.6 ± 0.2	0.3 ± 0.5	0.2 ± 0.1	0.1 ± 0.0
C 20:0	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	1.6 ± 1.2	1.1 ± 0.2	1.1 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.3 ± 0.3
C 20:1	1.0 ± 0.8	1.0 ± 0.2	0.8 ± 0.1	2.1 ± 1.2	2.1 ± 0.4	2.1 ± 0.5	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
C 20:2	1.2 ± 1.4	0.5 ± 0.7	1.1 ± 0.9	0.4 ± 0.3	0.5 ± 0.3	0.7 ± 0.3	0.4 ± 0.6	0.2 ± 0.1	0.1 ± 0.0
C 20:3(6)	0.8 ± 0.6	0.9 ± 0.3	0.9 ± 0.2	1.0 ± 0.5	1.2 ± 0.6	1.6 ± 0.5	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.3
C 20:4(6)	16.9 ± 5.7	21.3 ± 3.9	23.1 ± 4.9	18.5 ± 6.1^{b}	21.9 ± 4.6^{ab}	25.1 ± 2.6^{a}	11.5 ± 3.6^{b}	14.0 ± 3.5^{ab}	15.7 ± 2.6^{af}
C 20:3(3)	0.5 ± 0.2^{a}	0.3 ± 0.2^{ab}	0.2 ± 0.1^{b}	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
C 20:5(3)	0.6 ± 0.3	0.4 ± 0.3	0.5 ± 0.2	0.8 ± 0.3	0.7 ± 0.4	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.2
C 22:0	1.3 ± 0.7	1.0 ± 0.6	0.9 ± 0.2	0.7 ± 0.6	0.7 ± 0.5	1.2 ± 0.9	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.2
C 22:1(9)	0.4 ± 0.4	0.4 ± 0.4	0.8 ± 0.8	0.5 ± 0.5	1.1 ± 0.9	1.0 ± 1.1	0.3 ± 0.4	0.2 ± 0.1	0.2 ± 0.1
C 22:2(6)	1.2 ± 1.5	0.5 ± 0.2	0.7 ± 0.3	1.5 ± 0.9	1.6 ± 1.1	1.2 ± 0.5	0.4 ± 0.3^{a}	0.0 ± 0.0^{b}	0.0 ± 0.1^{bf}
C 22:4(6)	4.9 ± 2.8^{b}	7.8 ± 1.6^{a}	9.9 ± 2.1^{af}	5.8 ± 2.3^{b}	8.3 ± 2.9^{ab}	10.9 ± 2.2^{af}	0.3 ± 0.2	0.4 ± 0.2	0.6 ± 0.5
C 24:0	2.3 ± 1.9	1.4 ± 0.7	0.9 ± 0.5	1.4 ± 0.4^{a}	1.6 ± 0.7^{a}	0.7 ± 0.5^{b}	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
C 22:6(3)	7.4 ± 2.6^{e}	10.1 ± 0.9^{b}	12.7 ± 1.8^{df}	6.3 ± 1.9^{b}	9.2 ± 2.3^{a}	11.2 ± 1.2^{df}	2.0 ± 1.0	2.3 ± 1.1	2.7 ± 0.5
C 24:1(9)	2.5 ± 1.4	2.3 ± 1.5	1.5 ± 1.1	1.3 ± 0.7	2.6 ± 1.6	2.4 ± 2.3	0.6 ± 0.5	0.3 ± 0.1	0.2 ± 0.1
SFA[2]	44.7 ± 9.7^{a}	35.7 ± 5.9^{b}	31.2 ± 5.5^{bf}	41.6 ± 9.8^{a}	30.2 ± 7.1^{b}	26.7 ± 4.4^{bf}	58.6 ± 9.8	53.3 ± 8.8	50.4 ± 4.9
MUFA	15.7 ± 4.2	15.2 ± 2.5	13.2 ± 2.1	17.2 ± 3.0	17.9 ± 2.8	15.2 ± 2.4	13.6 ± 2.8	13.9 ± 2.1	13.4 ± 1.1
PUFA	39.4 ± 11.3^{b}	49.1 ± 5.4^{a}	55.6 ± 7.4^{af}	41.2 ± 11.5^{b}	51.9 ± 9.4^{a}	58.1 ± 4.2^{af}	27.8 ± 7.4^{b}	32.8 ± 7.0^{ab}	36.2 ± 4.2^{a}

[1] Means ± SD (% w/w) in 3 subclasses from 7, 8 and 7 animals, for the control, SM and GG group, respectively. Within a row, values with different superscript letters are significantly different at P< 0.05. Superscript letters with $^{a, b, and f}$ are significantly different at P<0.01, P<0.001, and P<0.0001, respectively.
[2] SFA, MUFA and PUFA represent saturated fatty acids, mono unsaturated fatty acids and poly unsaturated fatty acids, respectively.

FIG. 4

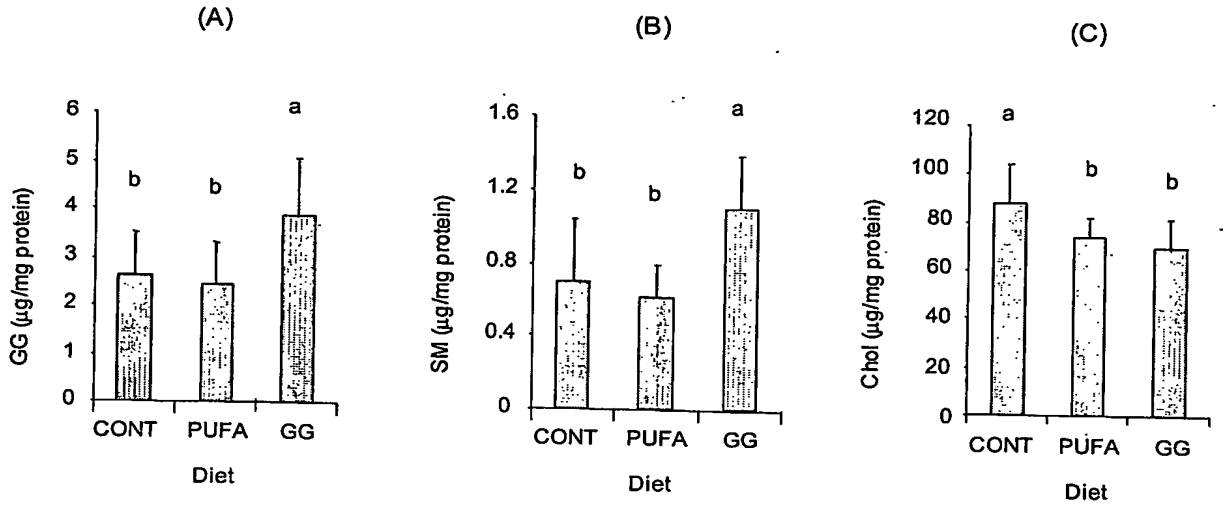
(A)
GG (µg/mg protein)
6
5
4
3
2
1
0
b
b
a
CONT
PUFA
GG
Diet
(B)
SM (µg/mg protein)
1.6
1.2
0.8
0.4
0
b
b
a
CONT
PUFA
GG
Diet
(C)
Chol (µg/mg protein)
120
100
80
60
40
20
0
a
b
b
CONT
PUFA
GG
Diet

FIG. 5

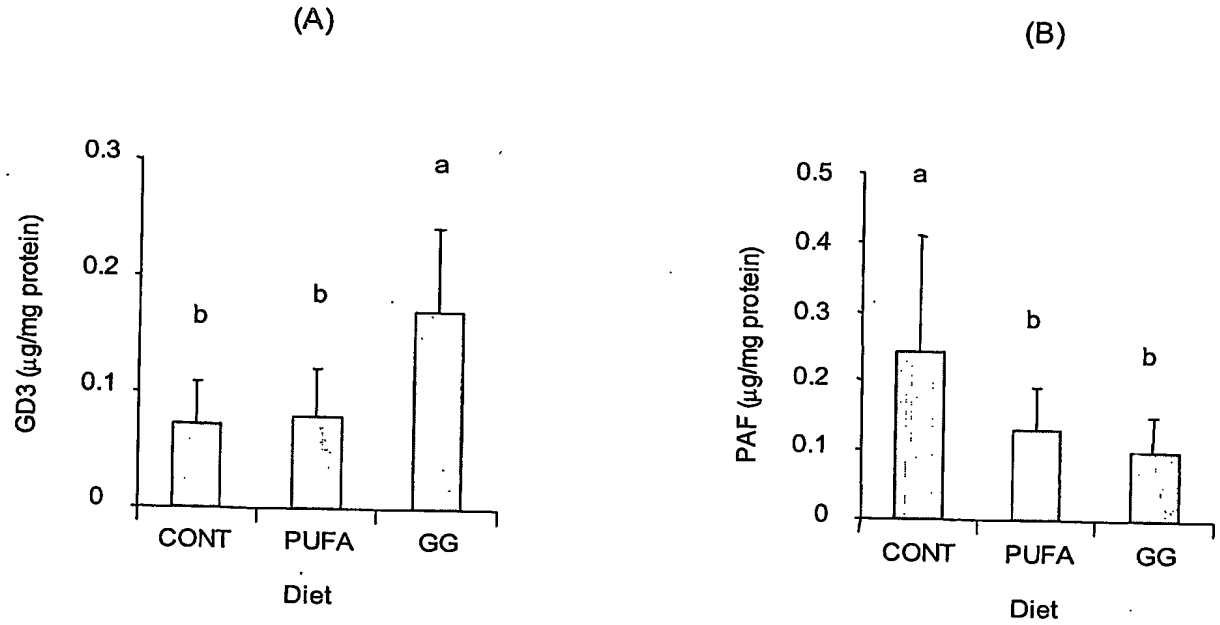
(A)
GD3 (µg/mg protein)
0.3
0.2
0.1
0
b
b
a
CONT
PUFA
GG
Diet
(B)
PAF (µg/mg protein)
0.5
0.4
0.3
0.2
0.1
0
a
b
b
CONT
PUFA
GG
Diet

FIG. 6

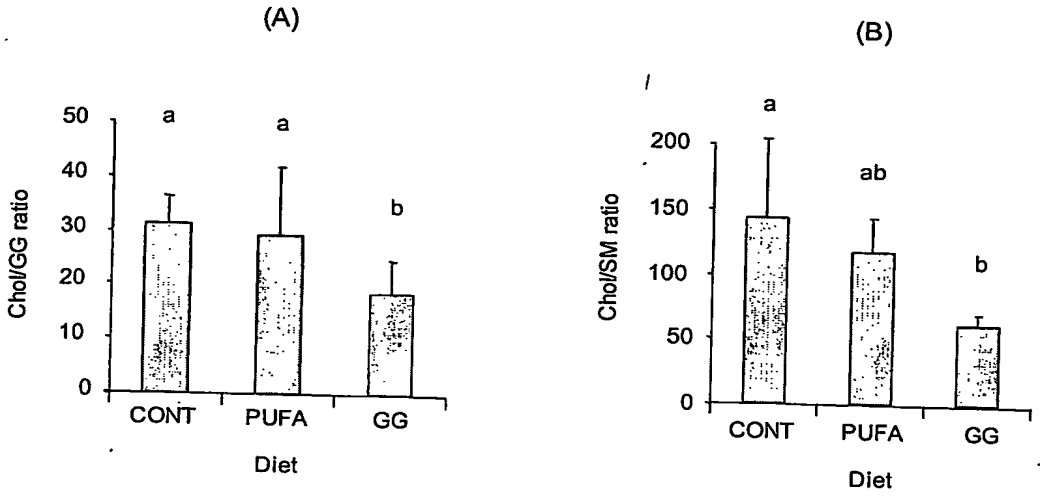
(A)
Chol/GG ratio
50
40
30
20
10
0
a
a
b
CONT
PUFA
GG
Diet
(B)
Chol/SM ratio
200
150
100
50
0
a
ab
b
CONT
PUFA
GG
Diet

FIG. 7

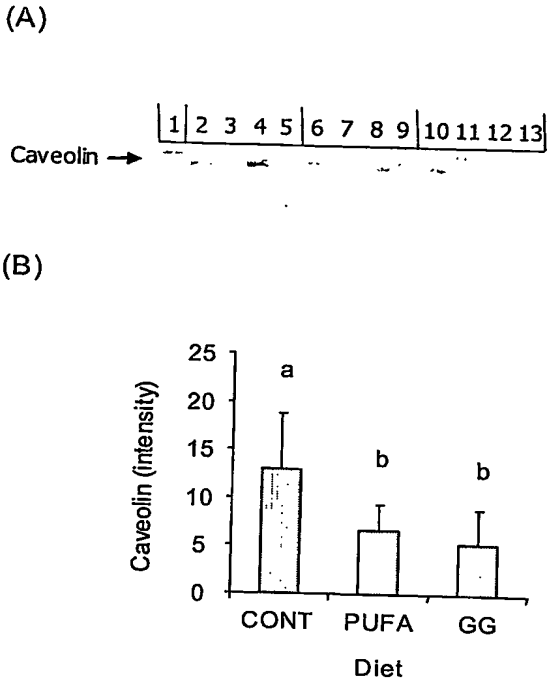
(A)
1 2 3 4 5 6 7 8 9 10 11 12 13
Caveolin
(B)
Caveolin (intensity)
25
20
15
10
5
0
a
b
b
CONT
PUFA
GG
Diet

FIG. 8

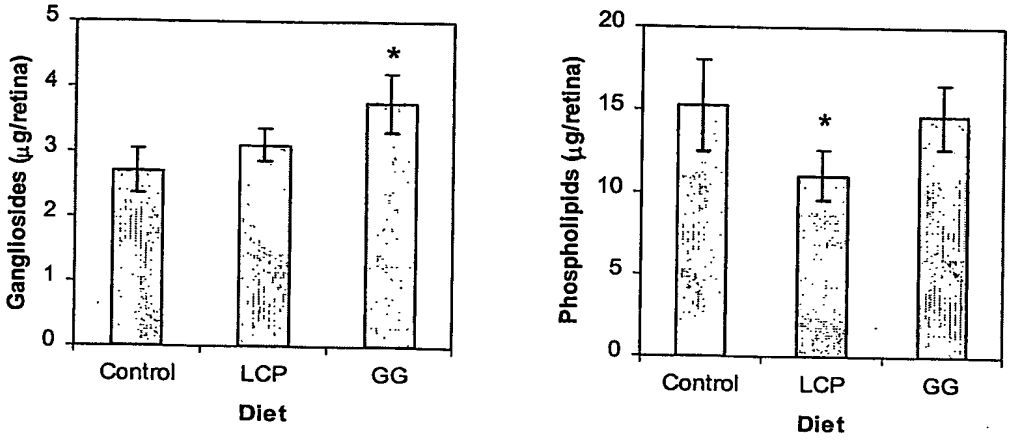
Gangliosides (µg/retina)
5
4
3
2
1
0
*
Control
LCP
GG
Diet
Phospholipids (µg/retina)
20
15
10
5
0
*
Control
LCP
GG
Diet

FIG. 9

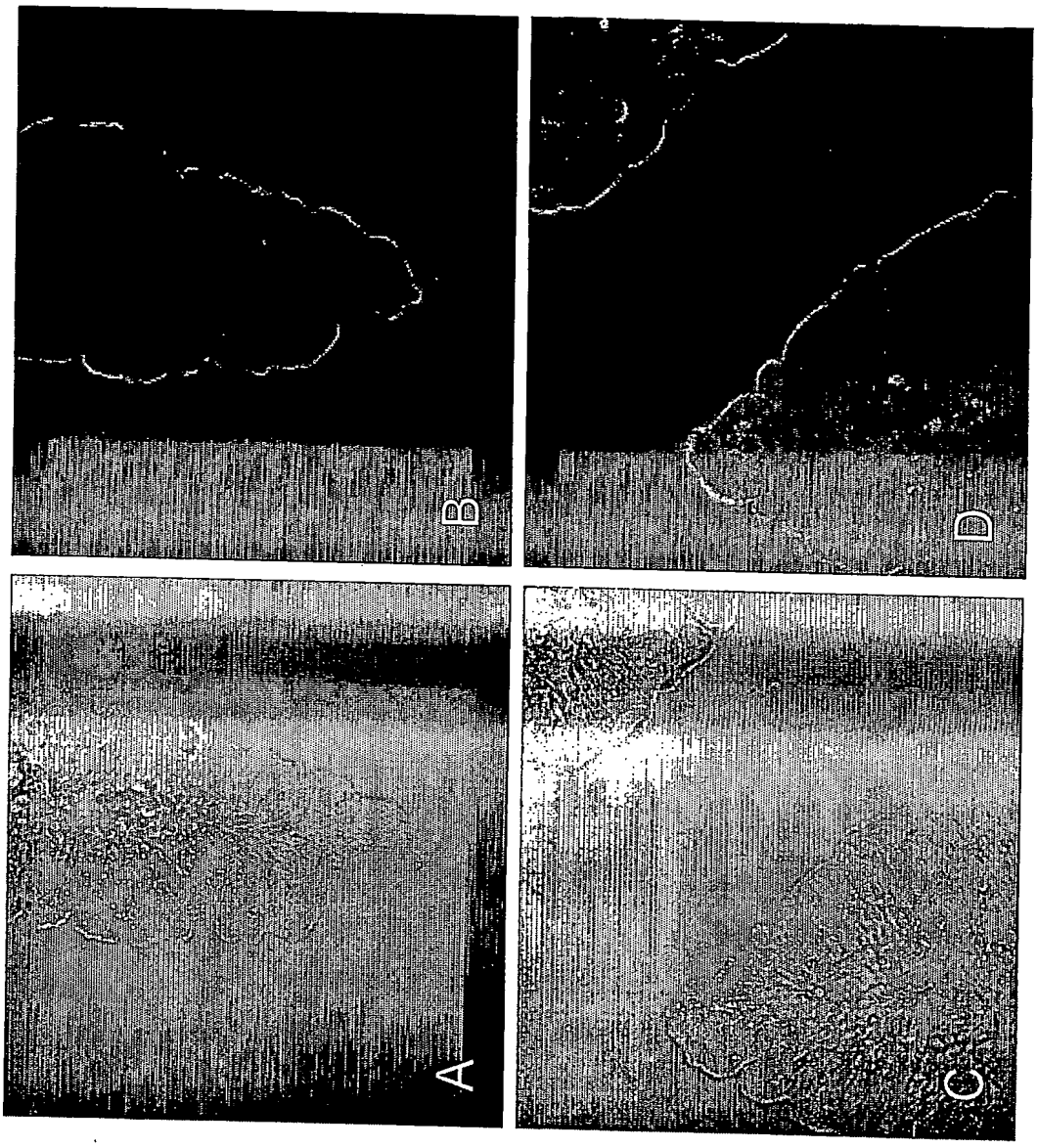

FIG. 10

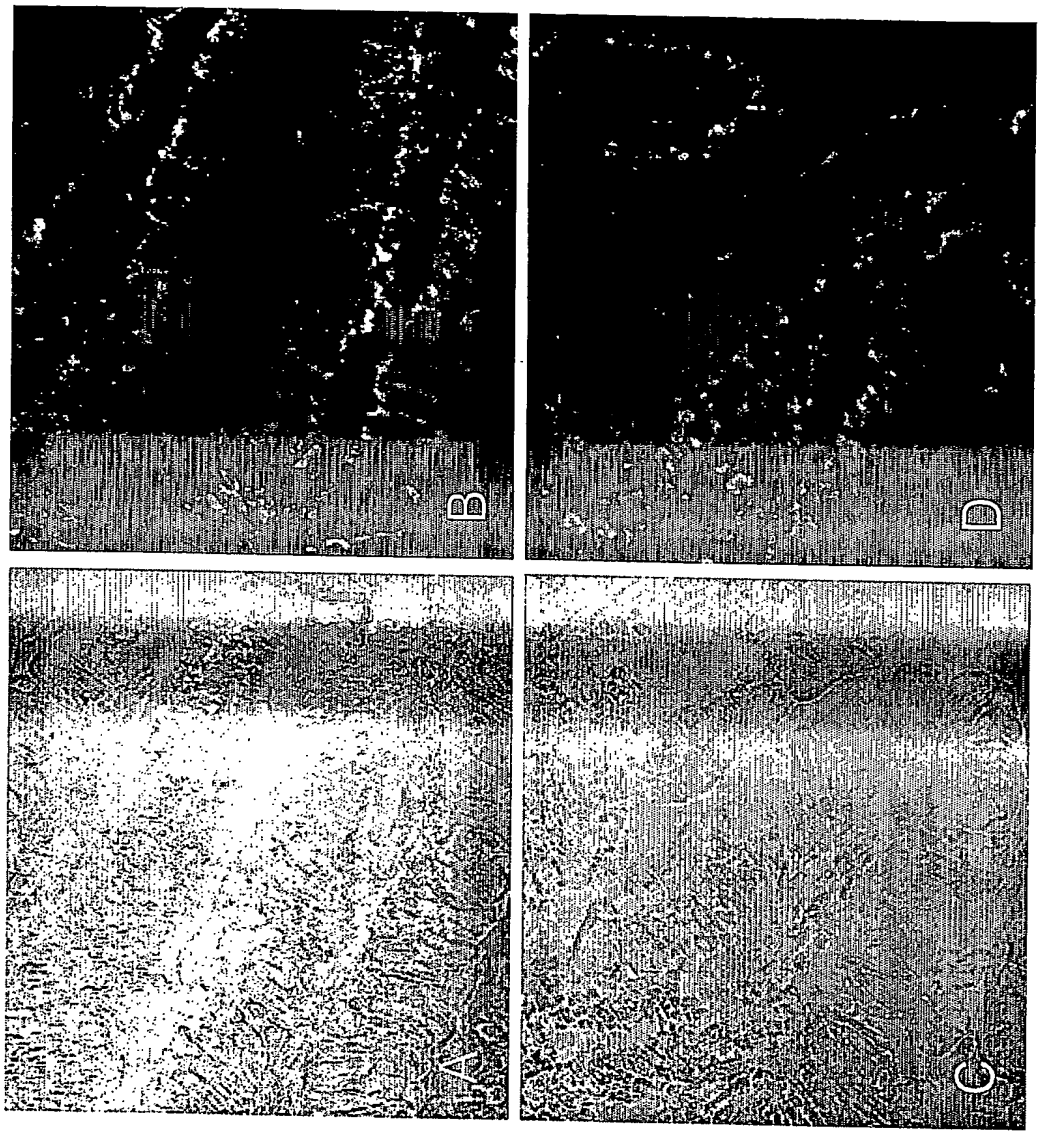

FIG. 11

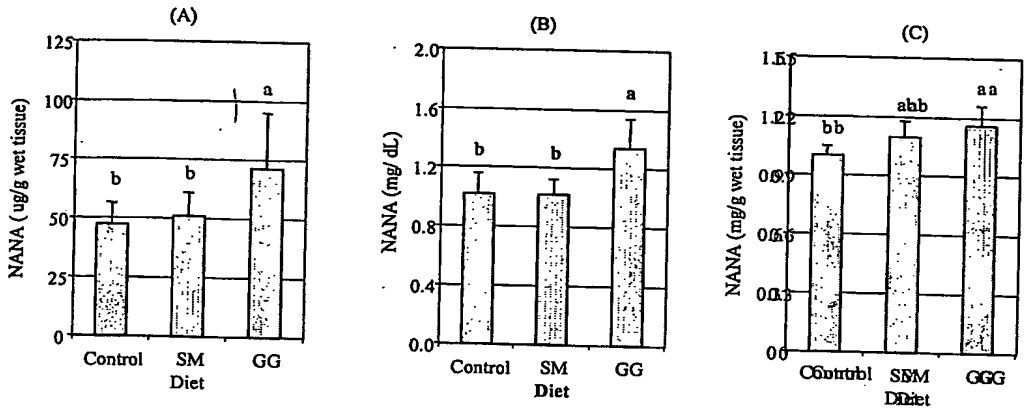
(A)
NANA (ug/g wet tissue)
125
100
75
50
25
0
a
b
b
Control
SM
GG
Diet
(B)
NANA (mg/dL)
2.0
1.6
1.2
0.8
0.4
0.0
a
b
b
Control
SM
GG
Diet
(C)
NANA (mg/g wet tissue)
aa
abb
bb
Control
SM
GG
Diet

FIG. 12

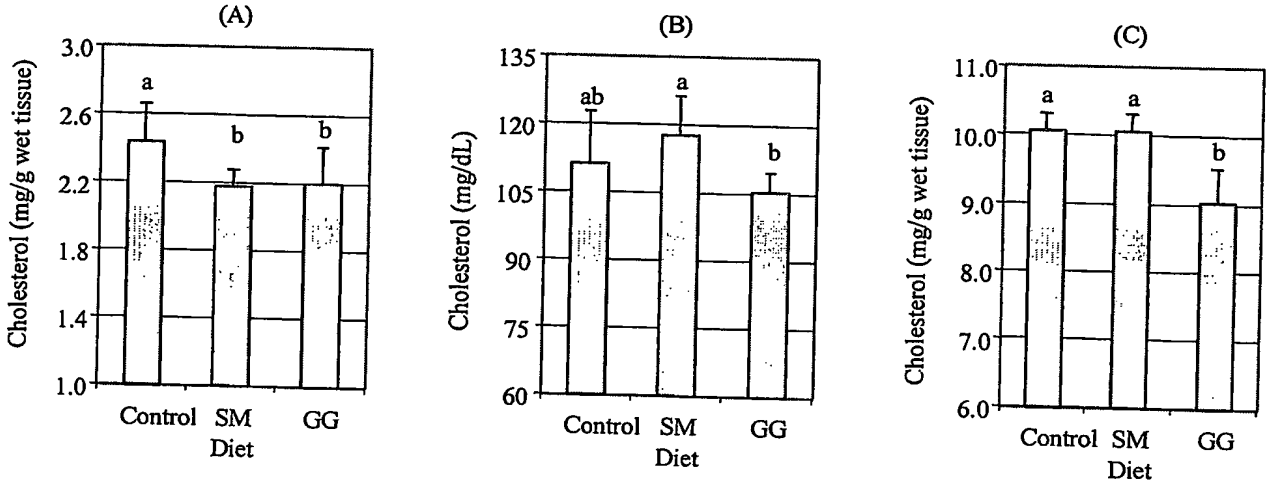
(A)
Cholesterol (mg/g wet tissue)
3.0
2.6
2.2
1.8
1.4
1.0
a
b
b
Control
SM
GG
Diet
(B)
Cholesterol (mg/dL)
135
120
105
90
75
60
ab
a
b
Control
SM
GG
Diet
(C)
Cholesterol (mg/g wet tissue)
11.0
10.0
9.0
8.0
7.0
6.0
a
a
b
Control
SM
GG
Diet

FIG. 13

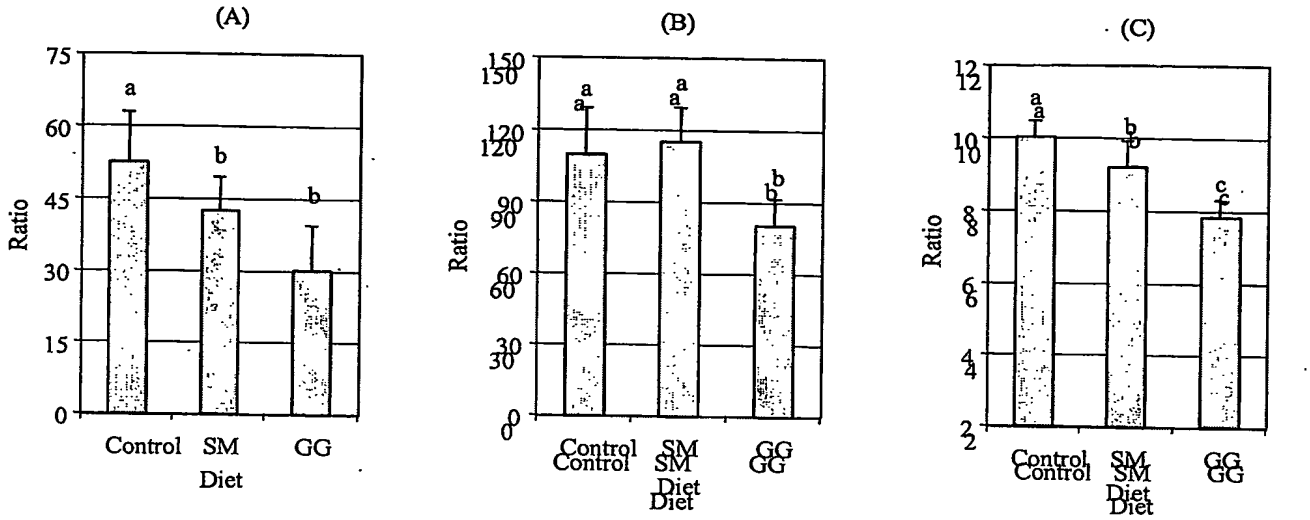
(A)
Ratio
75
60
45
30
15
0
a
b
b
Control
SM
GG
Diet
(B)
Ratio
150
120
90
60
30
0
a
a
b
Control
SM
GG
Diet
(C)
Ratio
12
10
8
6
4
2
a
b
c
Control
SM
GG
Diet

FIG. 14

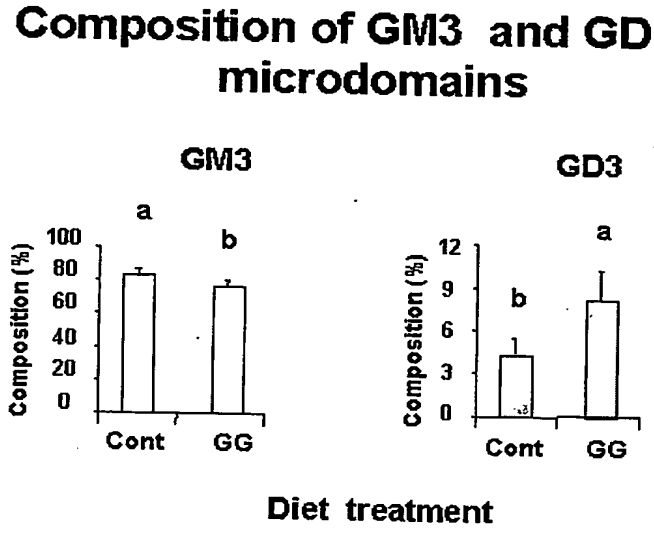
Composition of GM3 and GD3 in microdomains
GM3
GD3
a
b
a
b
Composition (%)
100
80
60
40
20
0
12
9
6
3
0
Cont
GG
Diet treatment

FIG. 15

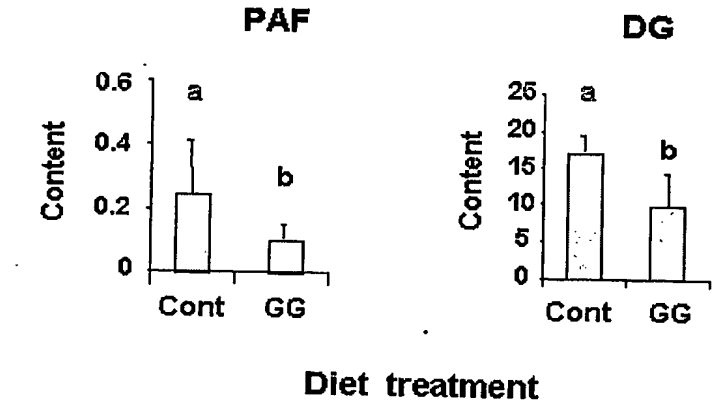
Content of PAF and DG in microdomains
PAF
DG
a
b
a
b
Content
0.6
0.4
0.2
0
25
20
15
10
5
0
Cont
GG
Diet treatment
Content is presented by µg/mg protein

FIG. 16

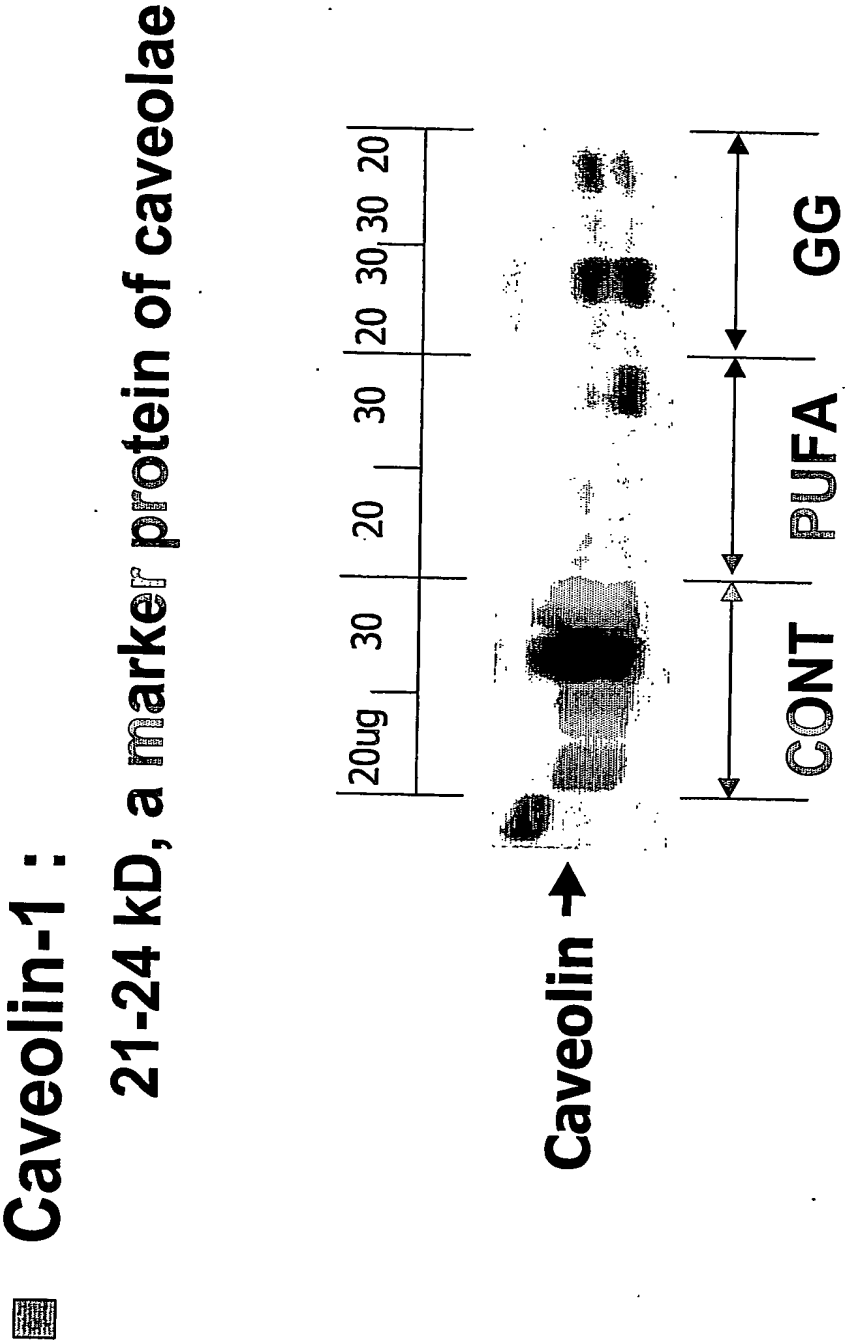
Caveolin-1 :
21-24 kD, a marker protein of caveolae
Caveolin
20ug
30
20
30
20
30
20
CONT
PUFA
GG

FIG. 17

Diet group/Statistical P value	Control	SM	GG	P
NANA (mg/DL)	1.02 ± 0.14^{b}	1.02 ± 0.10^{b}	1.34 ± 0.20^{a}	0.003
Phosphorus (mg/DL)	6.83 ± 0.71	6.92 ± 0.34	6.81 ± 0.22	-
Cholesterol (mg/DL)	111.3 ± 11.4^{ab}	117.8 ± 8.5^{a}	105.4 ± 4.2^{b}	0.03
Triglyceride (mg/DL)	94.7 ± 27.0^{ab}	107.1 ± 18.5^{a}	76.8 ± 10.5^{b}	0.02
NANA/P ratio (mg/mg)	0.15 ± 0.02^{b}	0.15 ± 0.02^{b}	0.20 ± 0.03^{a}	0.006
Cholesterol/NANA ratio (mg/mg)	110.1 ± 19.1^{a}	115.5 ± 14.1^{a}	80.4 ± 11.2^{b}	0.002
Cholesterol/P ratio (mg/mg)	16.26 ± 1.23^{ab}	16.97 ± 1.05^{a}	15.34 ± 0.72^{b}	0.06

FIG. 18